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THE EDITOR'S VIEWPOINT

Schlack, Flynn and Gerende (p. 265) contribute a paper from the Naval Medical Research Institute on the effect of age and region of birth upon the relative number of dental prosthetic replacements. Other papers have appeared from the Navy's research unit on this subject, but the material covered here is different. It represents an analysis of the dental records of some 70,000 navy men. Dental replacements appear to increase with age, as would be expected, although some types of crowns and bridges show a decrease beyond the 35 year mark. One of the most interesting parts of this study, and previous studies by Schlack and his associates, is the finding that people in the West-South-Central region have less need for dental care than those in other regions, and that the New England states show a particularly high rate of dental destruction. While some factors associated with these findings may come to mind at once, the underlying causes are a challenge to dental investigators and may present some of the most valuable clues in the subject of dental caries etiology.

From Denmark, H. Eggers Lura (p. 203) sends a study on salivary phosphates and phosphatases. The work is quite inclusive, as indicated by the results presented in 31 tables. Lura has shown that considerable shifts in phosphorus fractions result from the activity of phosphatases and that bacteria, tissue cells and other factors may influence the phosphatases. The finding that saliva, when left standing, may show marked shifts in the phosphorus fractions suggest the needs for revisions of many phosphorus presented in the past after analyses on saliva left for varying periods before study. As the author suggests, phosphorus analysis should be carried out immediately after the collection of saliva.

McClendon and Foster (p. 233) report their studies on the effect on dental caries of fluorapatite powders used to brush the teeth. McClendon's interest in fluorine dates back to 1917 when he observed the insolubility of fluoride in sea water. The teeth of rats brushed with powdered rock phosphate showed less decay than a comparable group of litter mate control rats. The material was also applied to the teeth of students and marked reduction was reported in the incidence of new cavities per man per year. An interesting part of the study was the lack of effect from swallowing one gram of rock phosphate (containing 31 milligrams of fluoride) daily for over a year. The authors believe that the toxicity of fluorine is greatly exaggerated except for the mottling produced in developing teeth. They believe that fluorides could be used effectively in brushing the teeth to reduce the incidence of dental caries.

Tulacek and Tilden (p. 247) studied the action of antibiotics, in vitro, on microorganisms isolated from root canals. Their findings are applicable beyond the limited field of root canal therapy. They found various resistance of these oral microorganisms to penicillin and streptomycin. Six of the streptococcal strains isolated showed marked resistance to penicillin and to streptomycin. It is interesting to note that 7 cultures of lactobacilli showed considerable resistance to penicillin and that when lactobacilli and streptococci were mixed the resistance of the mixture was very much greater than the resistance of pure cultures of either organism. Those hoping to use antibiotics for the control of dental caries should be very much interested in these results.

Swenson (p. 273) produced periodontal pockets in the mouths of dogs by pushing back the epithelial attachment and applying a copper band protruding into the area from which the gingiva had been surgically removed. Pockets produced by leaving the band on for 15 days were still present 150 days after the bands were removed. A

method for studying periodontal disease and the reattachment of epithelium to the tooth root is afforded.

Frisbie and Nuckolls (p. 181) have studied caries of enamel in carefully prepared sections of human enamel. They believe that caries operates beneath apparently sound and intact enamel surfaces. Although the authors show a series of photomicrographs in which they claim to demonstrate their 19 observations, we believe that there is still some doubt as to the validity of their conclusions. We believe that they are entirely justified in voicing their beliefs and we hope that by publishing this paper, further discussion of the problem will be stimulated. For example, in fig. 13 we believe that all of the organisms are not of coccal or spheriodal form. We believe that whether the caries is advancing through organic or inorganic material is largely a matter of interpretation of the photomicrographs or the original sections. Such pictures as presented in fig. 11 have been seen in many authors' illustrations and have been interpreted entirely differently. Many of the observations were made by W. D. Miller in his *Microorganisms of the Human Mouth* (Phila.: S. S. White), 1890. Miller said (p. 168): "The space between the loosened prisms are, in these cases, often filled with the same fungal masses, whereas the latter never penetrate between the prisms of normal enamel. In grinding, the masses of bacteria, as well as the loosened prisms, are generally torn away. After the enamel has once been perforated by decay, its further destruction proceeds principally from the inner surface. This statement may at first seem strange, but will be found on closer examination to be in accordance with the observed facts. We will find furthermore, in nearly all large cavities the decay extending from the dentine directly upon the inner surface of the enamel." Again on page 170 Miller wrote: "The whole looks as if the interprismatic substance were dissolved, i.e., the connection between the prisms destroyed." A very apt quotation from Miller appeared on page 211 of his work: "We must rid ourselves of the impression, . . . that the parasites of the human mouth makes holes in the dentine by boring into it as a worm bores into wood or by gnawing at it as a dog gnaws at a bone. Bacteria have no apparatus for boring, nor do they have mouths or any provision for breaking off small portions of solid substances which they then swallow whole or take directly up at any point of their periphery after the manner of an amoeba. They nourish themselves alone by substances in a state of solution, and if we present them solid substances they themselves must liquefy them before they can make any use of them for their own nourishment."

Pindborg (p. 261) has studied the effect of tobacco on gingivitis and on calculus formation. The study was made on a group of Danish Marines. The author believes as a result of this study, that the custom of forbidding smoking during treatment of Vincent's infection should be continued. He also claims that one who smokes has a greater chance of developing ulceromembranous gingivitis and that deposition of calculus is effected by consumption of tobacco. The author is to be congratulated on this study which was made under extremely advert conditions during the wartime in Europe.

Volker and Pinkerton (p. 225) have studied the factors influencing oral glucose clearance by having the individuals consume a glucose wafer, dissolved by sucking, a glucose cube eaten at normal rate, a solution of glucose, and gum base glucose chewed during an experimental period. From their studies, it appears that the clearance from the mouth of portions of glucose varies with different methods. The clearance was found to be most rapid for the rinsed and swallowed glucose and slowest for the sucked glucose. While this is important as far as the retention of glucose which might be available to the acidogens of the mouth is concerned, after the microorganisms in the plaque have once produced acid there is little likelihood that they would be able to receive more glucose because of the precipitated proteins in the mass which form an impermeable membrane during the time that they are at acid level.

In another report, Volker and Pinkerton (p. 229) give the relative acid production in saliva of different carbohydrate mix-

tures. They found that acid production in sucrose-saliva and starch-saliva mixtures is comparable to that of glucose-saliva mixtures. It is suggested that sucrose and starch may not be broken down into glucose before their degradation in the oral cavity. Raw carbohydrates were shown to be converted to acid by saliva at rates similar to those observed for refined sugars. These observations should be interesting to those who have for a long time believed that consumption of refined carbohydrates should be discouraged, but that natural carbohydrates can be used to take their place to reduce the possibility of decay.

Faulkenheim and Hodge (p. 241) used bone powder samples marked with radioactive phosphorus (P 32) to trace the mechanism of fluoride fixation. Their observations suggest that the mechanism of fluoride adsorption does not involve exchange with phosphate since no more tagged phosphorus went from bone to solution after fluoride adsorption than in the absence of fluoride ions.

H. B. G. R.

CARIES OF THE ENAMEL

A Histopathological Study of Caries of the Human Enamel Operating beneath Apparently Sound and Intact Enamel Surfaces[1]

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INTRODUCTION

A previous histological study on amelogenesis (1, 2) in the molar tooth of the rat clearly demonstrated that each enamel-forming cell is responsible for the formation of a single organic rod and its adjacent inter-rod substance which subsequently undergoes a progressive mineralization. Saunders, Nuckolls and Frisbie were able to show that this protein matrix remained within the enamel up to the time the tooth erupted. It was, therefore, reasonable to assume that it might be recovered from the adult structure if the proper procedures were employed.

Frisbie, Nuckolls and Saunders (3) reported the presence of an organic matrix in the adult human tooth and described a method for its recovery. The demonstration of a continuous organic structure in the adult tooth greatly modified many of the existing theories of enamel formation and, in addition, provided a valuable means by which a more direct observation of the mechanisms involved in the pathology of caries of enamel can be made. In a report (3) of the carious lesion and its effect on the matrix, they claimed that caries of the enamel is more than a simple acid erosion effecting a removal of the inorganic salts and that organic matrix is fundamentally related to the pathological process. A working hypothesis, based on these observations, was offered: namely, that with the establishment of the lesion, caries is a degradation of the organic matrix resulting from the enzymatic action of microorganisms, which is followed by a physical disintegration of the inorganic salts. This disintegration is in part accounted for by the loss of the supporting matrix.

Since reporting these findings, further studies have been conducted on the histopathology of the carious lesion on macroscopically smooth, unbroken enamel surfaces in which "brown stain" on the enamel surface was the only visible evidence of the presence of a caries lesion (4).

MATERIAL AND METHODS

Following the identical technic reported in earlier studies, human teeth were placed in 10% neutral formol immediately following their extraction. These

[1] Presented at the 25th General Meeting of the International Association for Dental Research, Kansas City, Missouri, March 17, 1946 (*J. D. Res.* 25: 166, 1946). This work was supported in part by a grant from the James Foundation of New York, Inc. Received for publication March 13, 1947.

teeth were allowed to remain in the solution for a minimal period of 30 days. When observed under the binocular microscope, the teeth selected for study showed evidence of discoloration in confined areas only, with no visible evidence of a break in the surface continuity. These areas on the enamel surface, although discolored, were essentially hard. Longitudinal sections, approximately 1 mm. in thickness, were cut through these areas under water, care being taken to include on either side apparently sound, nondiscolored enamel. These thick sections were examined under the binocular microscope in order to provide a gross picture of the carious penetration. Since it was conceivable that varying amounts of dentin might be involved, a sufficient amount of this structure was retained in order to study the relationship of caries of the enamel to that of dentin. It was important to keep the specimen wet at all times, since a slight dehydration produces artifacts and shrinkage, especially at the dentino-enamel junction. The sections containing the carious areas were fixed for a further 60-day period or longer. Decalcification was then carried out in 10% nitric acid in 80% alcohol. After decalcification of the matrix, the technic employed for embedding and sectioning of the tissue was essentially the same as that described by Frisbie, Nuckolls and Saunders (3).

Stains employed included the use of hematoxylin and eosin, which give a general differentiation between the carious and non-carious organic matrix. Gram-Weigert and MacCallum's modification of Gram stain were found most suitable for the demonstration of microorganisms. The general disadvantage of the Gram stains is their tendency to fade rather rapidly; therefore it is necessary to photograph the sections in color almost immediately following their preparation. Von Kossa's (5) method of staining for calcium was employed in order to determine whether a complete removal of the calcium salts from the matrix had been accomplished. Serial histologic sections, cut to the order of 3 and 4 μ were made and every tenth section was photographed.

OBSERVATIONS

A. *Restricted Caries* (So-called arrested caries).

The term "restricted caries" is applied for convenience to designate a mode of penetration which is slowly progressive and is evidenced by the appearance of a brown-stained spot in the intact enamel surface of a tooth (*fig. 1*). The pathological process does not differ essentially from that described previously (3).

1. *The Macroscopic Appearance of the Surface Lesion in Restricted Caries.* The gross appearance of the surface lesion is that of a hard, intact enamel surface, a part of which contains a stained area which varies in intensity from yellow to brown. Thin ground sections demonstrate the discoloration extending from the surface to varying depths within the enamel structure. In areas of more extensive involvement, the brown stain may extend to the dentino-enamel junction and may involve the dentin (*fig. 2*). In these undecalcified ground sections, it should be emphasized that, although discolored, the enamel on the surface, as well as the carious extension within the enamel, is essentially hard in

the physical sense. Examination by means of instruments may or may not reveal a roughened or irregular surface (*fig. 1*).

2. *The Microscopic Appearance of the Surface Plaque.*[2] It is evident that the initial surface lesion has been established for some time. A microscopic plaque overlies the stained carious surface of the tooth. The surface appears unbroken in situ, but when the enamel is fixed, decalcified, sectioned and studied under the higher powers of the microscope, evidence of numerous microscopic portals of entry can be determined. Spheroid-appearing microorganisms have long since penetrated the surface and established a lesion well within the enamel. Gram-positive filamentous organisms now form a dense mat on the surface of

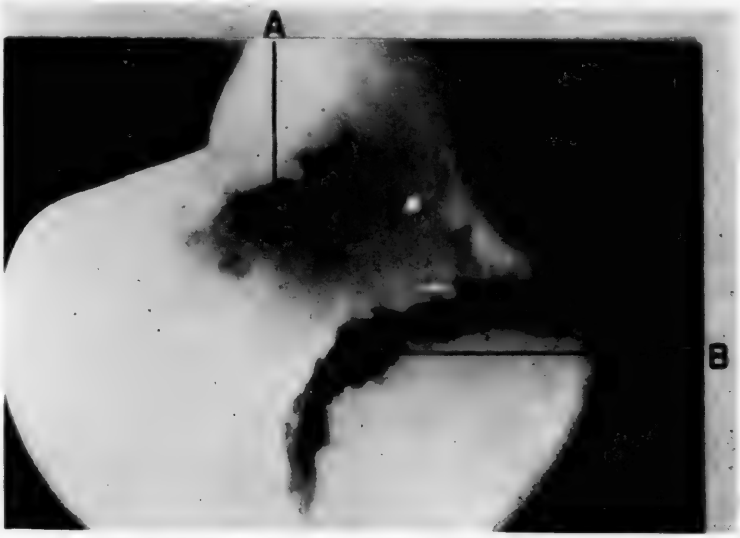

Fig. 1. Approximal surface of an adult human lower molar tooth × 5. Illustrating brown-stained spot in hard, intact surface (Restricted caries, or so-called arrested Caries). A. Brown-stained area of hard, intact enamel surface. B. Calculus adhering to cervical line.

the tooth and have a parallel arrangement to one another (*fig. 3*); in the deeper part, approximating the disintegrating surface of the tooth, these filamentous forms are seen to project themselves directly into the surface lesion so as to appear partly within the degraded organic mass (*fig. 4*). Their appearance within the matrix is apparently secondary to the establishment of the superficial carious process and is made only after some surface enamel degradation has taken place. The primary invaders are Gram-positive and appear to be of a spheroidal type.

3. *The Microscopic Appearance of the Organic Matrix in the Carious Lesion.* Although the surface of the tooth may seem apparently intact, a histologic examination of the serial sections taken from the brown-stained area will, in

[2] See reference 3, Plaque Formation.

many cases, reveal an organic disintegration of the matrix which extends in some instances to the dentino-enamel junction and there establishes a secondary focus and retrograde spread of the carious lesion (*figs. 2, 5*). This area of organic degradation may have had its surface initiation from multiple points on the

Fig. 2. Demineralized organic matrix from adult human tooth. 4 μ neutral formol fixation Hematoxylin and Eosin × 60. Note area of extensive involvement extending laterally within enamel and involving dentino-enamel junction. A. Demineralized normal enamel matrix. B. Lateral extension within enamel. C. General area of organic matrix degradation. D. Islands of uninfected normal matrix. E. Lateral and retrograde spread on enamel side of dentino-enamel junction. F. Initial penetration of carious process into dentin. G. Normal dentin.

tooth surface, but now the appearance of the early lesion is that of a solid front extending for a variable distance within the enamel (*fig. 6*). With deeper penetration, the carious process extends laterally in all directions beneath the surface of the tooth so that an involvement of a considerable amount of the enamel structure occurs (*figs. 2, 7*), therby establishing a central focus of infec-

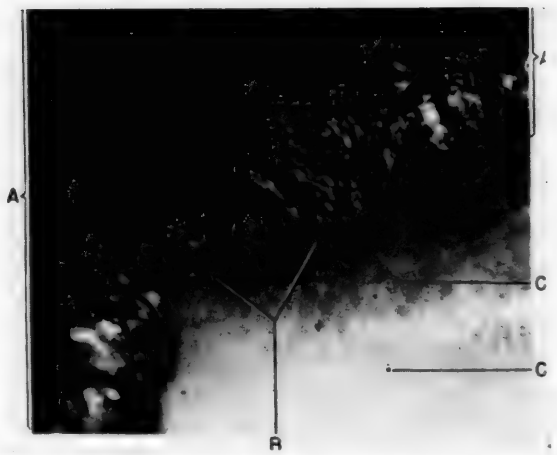

Fig. 3. Demineralized organic enamel matrix from adult human tooth. 4 μ neutral formol fixation MacCallum's Gram × 760. Note Gram-positive filamentous organisms in parallel arrangement to one another, forming characteristic dense mat of microscopic plaque in restricted caries. A. Filamentous microorganisms. B. Microorganisms in proximity to enamel matrix. Note evidence of numerous microscopic portals of entry and microscopic irregularities of surface. C. Normal enamel rods in cross section.

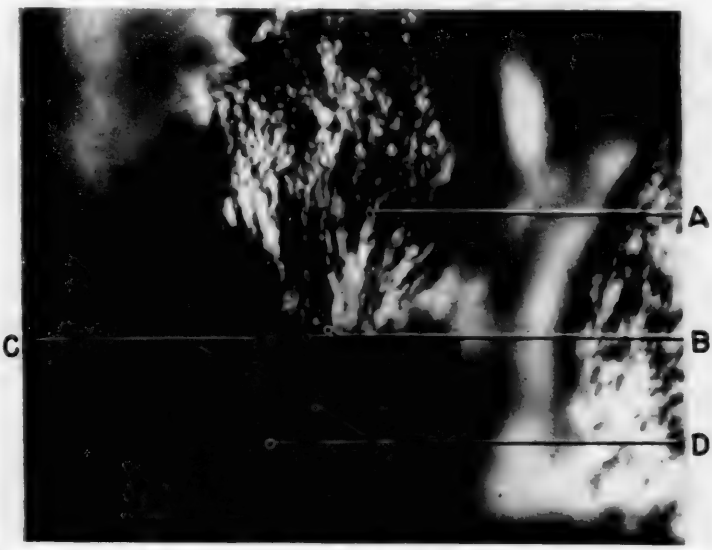

Fig. 4. Demineralized organic enamel matrix from adult human tooth. 4 μ neutral formol fixation MacCallum's Gram × 1140. Note surface degradation of organic matrix with filamentous forms projecting themselves directly into surface lesion so as to appear partly within degraded organic mass. A. Filamentous organims of surface plaque. B. Degraded organic matrix with filamentous micro-organisms partially within mass. C. Affected enamel rods in parallel relation. D. Inter-rod substance.

tion (*fig. 8*).[3] Multiple, irregular channels spearhead out in all directions from the periphery of this focus and extend through the substance of the healthy matrix (*fig. 9*). These spearheading tracts multiply and present extremely complex patterns as progressive degenerative changes occur and wide areas of the matrix become involved. Concomitantly with the spearheading tracts, the peripheral matrix of the central focus undergoes a progressive degeneration. Islands of healthy matrix are isolated by the anastomosing of these carious channels.[4] When this process reaches its final stage, all degrees of solution of the organic rods may be observed in the center of the lesion (*fig. 10*).

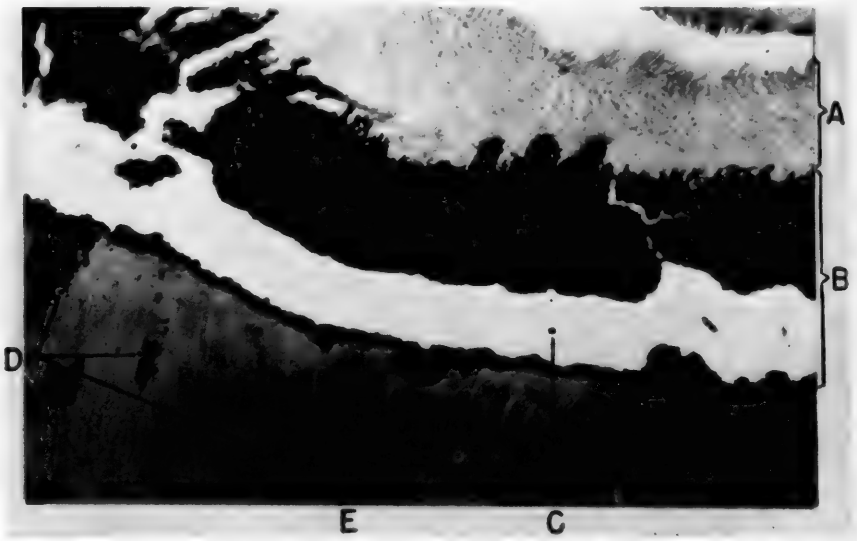

Fig. 5. Demineralized organic enamel matrix from the adult human tooth. 6 μ neutra formol fixation MacCallum's Gram × 240. Note extension of carious lesion to dentino-enamel junction with lateral extension on enamel side and retrograde spread. A. Normal enamel overlying carious lesion. B. Carious lesion and retrograde extension toward surface of tooth. C. Artifact due to shrinkage in preparation. D. Invasion of occasional dentinal tubule by Gram-positive spheroidal microorganisms. E. Normal dentin.

4. *The Appearance of the Organic Matrix at the Periphery of the Focus.* The appearance of the pathological process associated with the peripheral extension of the lesion in restricted caries differs but little from that of the lesion of superficial caries, which was described by us in a previous communication (3). It is to be pointed out, however, that the ends of the enamel rods have been opened up and, occasionally, organisms are found *spearheading* deep within the matrix, well beyond the general area of decomposition, and occupying the core of the individual rods or lying within the inter-rod matrix. In such areas of deeper

[3] See reference 3, *fig. 7.*
[4] See reference 3, *fig. 11.*

penetration, the organisms seem to be of the spheroid form and take the Gram-positive stain (*fig. 9, 11*). The protein matrix which has been invaded by microorganisms is strongly acidophilic and contrasts markedly with the surrounding normal matrix. This increase in the staining reaction is the earliest indication of a pathological change occurring in the matrix. The increasing acceptance of the dye by the matrix may extend into the substance of the

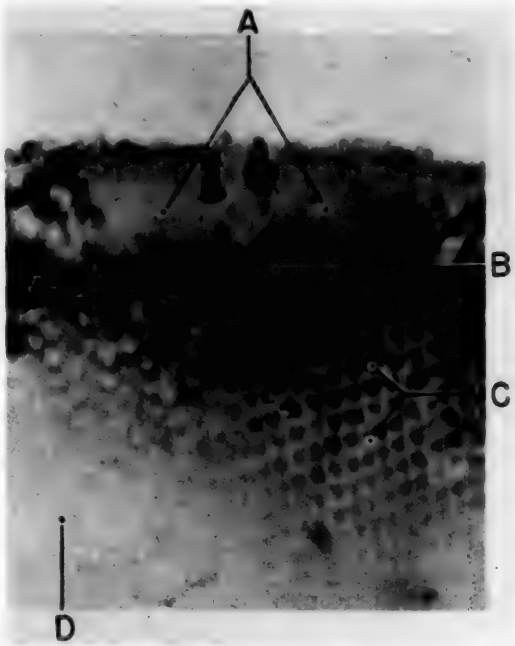

Fig. 6. Demineralized organic enamel matrix from adult human tooth. 4 μ neutral formol fixation Gram Weigert × 600. Note appearance of early lesion extending for variable distances within the enamel. A. Bacterial plaque and organic matrix which has undergone complete degradation. B. Intense acidophilic staining of infected organic rods. C. Extension of infection into deeper part of enamel matrix. D. Normal enamel rods on cross section.

enamel for a considerable distance ahead of bacterial invasion and disintegration of the enamel.[5]

The periphery of the matrix in the infected area of the central focus, as well as the periphery of the isolated islands of normal structure, presents a similar increase in staining reaction. This is closely followed by a progressive loss of structural detail of the organic rods toward the center of the focus, and when this alteration is advanced, the matrix becomes almost homogeneous in appearance (*fig. 10*). Eventually the affected peripheral area undergoes a complete breakdown. The relatively early stages of degradation present an intensely staining

[5] See reference 3, *fig. 4*.

matrix, and those areas at the periphery of the lesion where the rods have just begun to lose their definition are, for the greater part, free of microorganisms. At a later stage, when the organic rods have begun to undergo a definite disintegration and solution, microorganisms are observed in great abundance. It should be emphasized that, at this stage of extensive involvement, the organisms are found predominately at the periphery of the lesion. The microorganisms associated with the early invasion of the enamel matrix have a spheroidal appearance. When the degradation of the matrix is more advanced,

Fig. 7. Demineralized enamel matrix from adult human tooth. Neutral formol fixation hematoxylin and eosin × 40. Note lateral extension of advancing tracts from central focus leaving shell of uninvolved enamel on surface of tooth. A. Uninfected enamel matrix forming surface of tooth. B. Lateral extension of advancing tracts undermining surface enamel. C. Lateral and retrograde spread on enamel side of dentino-enamel junction. D. Artifact due to shrinkage during preparation. E. Invasion of dentin following establishment of central focus and retrograde spread of carious process. F. Normal dentin.

organisms found in the central focus show variation in morphological types. The spheroidal forms no longer predominate, but filamentous organisms are seen in greater abundance. Their relationship to the advancing lesion in the central focus is relatively constant in restricted caries (*fig. 12*). In the center of tne focus, where the breakdown of the organic matrix nears completion, relatively few organisms are to be found (*fig. 10, 12*).

5. *Retrograde Spread from Dentino-Enamel Focus.* Having reached the dentino-enamel juuction, the carious process extends laterally along the junction, opening up the dentinal extremities of the rods and inter-rod intervals, and thus establishes a retrograde spread. The lateral spread of the focus seems to be at

first confined to the enamel matrix, and only when disintegration is advanced does the dentin become involved (*fig. 5*). This spread on the enamel side of the junction appears to be a characteristic feature of the late phase of the carious lesion, although there is as yet no macroscopic evidence of physical disintegration of the enamel surface.

B. *Caries Penetration into the Dentin.*

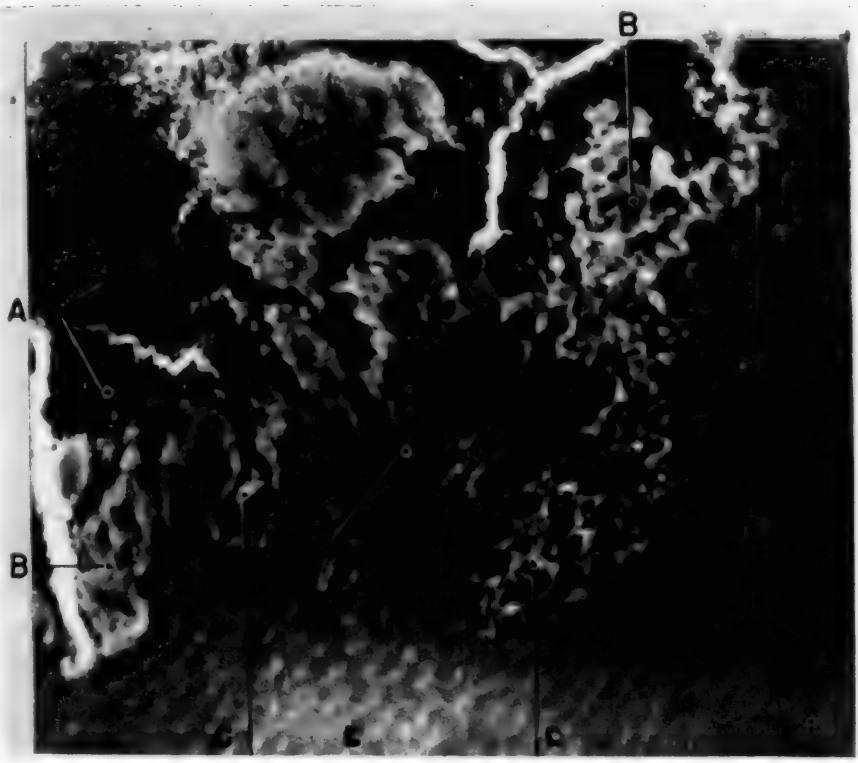

Fig. 8. Demineralized organic matrix from adult human tooth. 4 μ neutral formol fixation Gram Weigert $\times$ 1140. Note deep infection of organic matrix with numerous portals of entry from surface. Note first evidence of establishment of central focus of infection. A. Bacterial plaque. B. Degraded organic matrix. C. Multiple portals of entry. D. Beginning establishment of a central focus of infection. E. Normal organic rods of the matrix on cross section.

Concomitant with the establishment of a deep focus within the enamel structure and the lateral spread of the carious process on the enamel side of the dentino-enamel junction, microorganisms are observed to penetrate into the dentin (*fig. 5*). These microorganisms are of the same morphological type as those seen in the spearheads of the early enamel lesion. They are few in number at first and penetrate the dentin only after an amount of destruction has taken place on the enamel side of the dentino-enamel junction. These microorganisms

apparently follow along the inner wall of the dentinal tubule and are found in the center of the tubule only after the dentinal fibril has become affected (*fig. 13*) (6). The pathological process apparently progresses with greater rapidity in the dentin than it does in the enamel because of the higher content of organic material in the former, but the carious lesion and the microorganisms associated

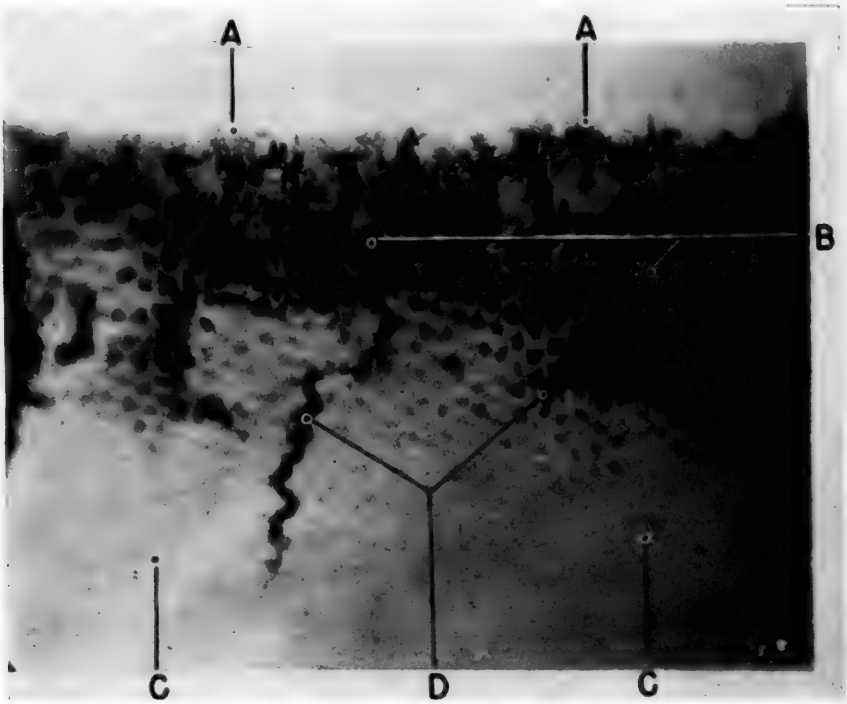

Fig. 9. Demineralized enamel matrix from adult human tooth. 4 μ neutral formol fixation Gram Weigert × 600. Note spearheading of infectious process from periphery of central focus into organic substance of healthy matrix. A. Perimeter of central focus of infection. B. Infected rods undergoing organic degradation, indicated by increasing acceptance of dye. C. Normal rods of enamel matrix on cross section. D. Spearheading of infectious process into substance of healthy matrix.

with the disease are essentially the same in both structures. A more complete description of caries of the dentin will be the subject of a further communication.

DISCUSSION

The action of fixative agents on the hard structures of the teeth has not been given sufficient emphasis in the past, and this lack of understanding has led to many misconceptions with regard to solubility and insolubility of enamel in both the developing and the erupted tooth. The nature of the fixative agent and its action on the matrix and the degree of mineralization of the matrix are

important factors which must be considered if experiments are to yield the true nature of the conditions at hand.

Several investigators, Baumgartner (7), Fleischmann (8), Malleson (9), Bodecker (10, 11), Bodecker and Bodecker (12), Bibby (13) and Berke (14, 15) have demonstrated penetration of bacteria in caries of the enamel and have also recognized that the disease is not entirely one of decalcification. They consider

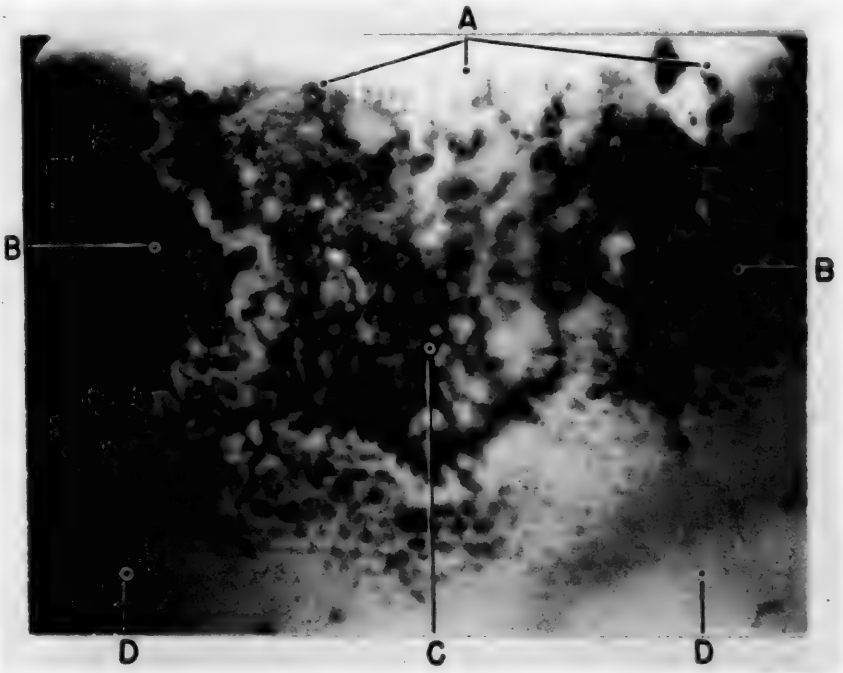

Fig. 10. Demineralized organic matrix from adult human tooth. Cross section of brown-stained area. 4 μ neutral formol fixation. Gram Weigert $\times$ 600. Note advanced degenerative carious process in which all degrees of matrix degradation may be observed. A. Surface of intact brown-stained area. B. Infected rods undergoing degradation at periphery of lesion. C. Central area of lesion where all rod detail is lost. D. Normal enamel with organic rods in cross section. Note: Clinically, surface continuity of enamel is relatively intact and may show only slight roughening.

caries to be a two-phase process in which both acid decalcification and proteolysis play a part. Careful study of the papers of these investigators fails to reveal that they were aware of a complete organic matrix within the enamel and of its importance in the overall pathological picture. Since necessity for complete denaturation during fixation was not appreciated at the time of their investigations, they were unaware that the organic content of the enamel was of importance in the carious process.

Beust (17) discussed the clinical appearance of brown stains on the surfaces

of teeth and demonstrated to his satisfaction, by laboratory procedures, that these areas contained some organic material. He was able to demonstrate an organic matrix in these areas by incompletely decalcifying the enamel. It was noted that the mass in brown-stained areas remained relatively intact. His next procedure was to ash the stained area and then complete the demineralization. By ashing he destroyed the organic part and the remaining salts went

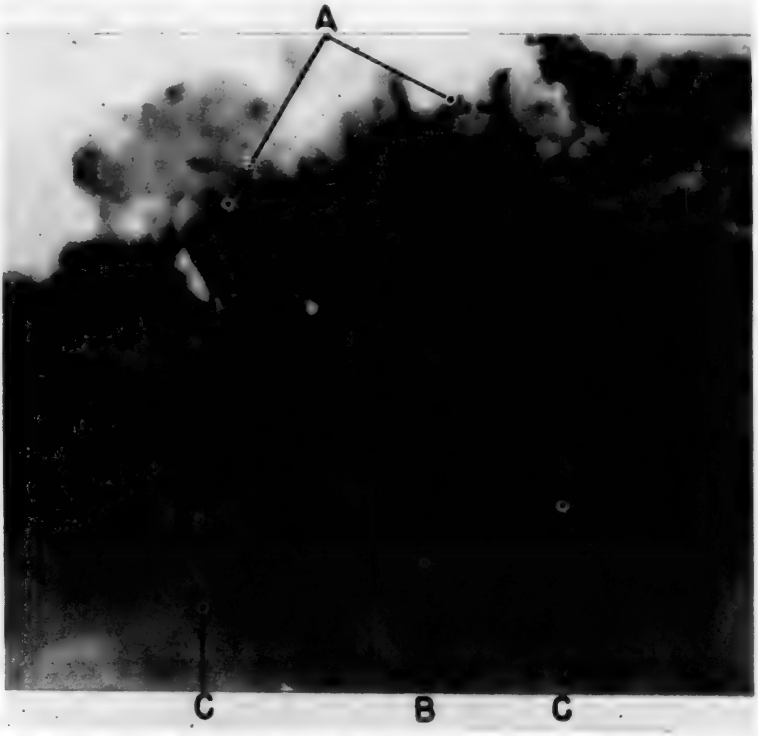

Fig. 11. Demineralized organic enamel matrix from adult human tooth. 4 μ neutral formol fixation. MacCallum's Gram × 1140. Organisms are of spheroidal type and occupy cores of individual rods or inter-rod substance. A. Degraded perimeter of lesion. B. Deeply penetrating Gram-positive, apparently spheroidal organisms within rod. C. Normal enamel matrix.

into solution because of the acid action. Beust likewise appears to have been unaware of the problems of fixation in preserving the organic content of the enamel; however it might be assumed that he employed a fixing agent as a more or less routine procedure so that a partial fixation of the organic substance would allow for a recovery of the carious matrix in a specific area.

In 1932 Enright, Friesell and Trescher (18) repeated the work of Beust. In so doing they employed a fixing agent prior to decalcification of the enamel and succeeded in recovering a limited amount of the protein from the stained

area; but they, too, were apparently not cognizant of the importance of the organic matrix in the development of enamel and in the study of the pathology of enamel. They offered as an explanation that this reaction was the result of an accumulation of mucous, dead organisms and debris. This appearance was called condensation or consolidation, a term used in much the same sense as designated by Malleson (9) Bodecker (10, 11, 12) and Bibby (19, 20) who thought of condensation as a defensive mechanism which in some way rendered

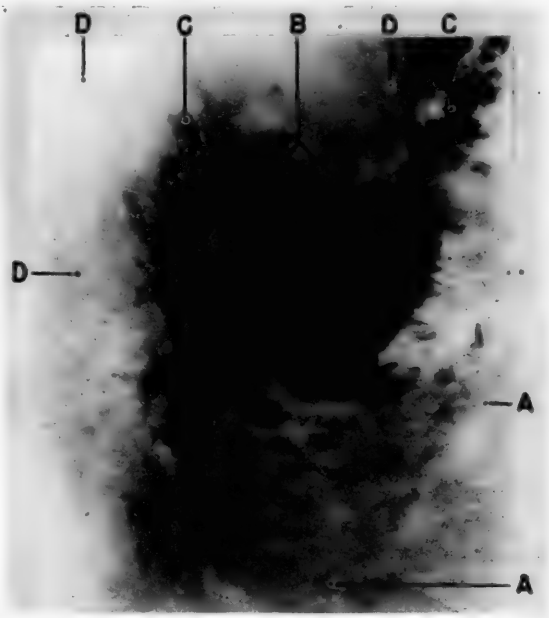

Fig. 12. Demineralized organic enamel matrix from adult human tooth. Cross section of periphery of central focus of advanced lesion. Neutral formol fixation 4 μ Gram Weigert $\times$ 750. Note position and character of organisms at stage of extensive involvement. A. Central focus of carious lesion. B. Filamentous microorganisms at periphery of advanced carious lesion. C. Organic matrix undergoing breakdown ahead of organisms. D. Unaffected enamel matrix. Note: At this late stage of matrix degradation, microorganisms are found predominantly at periphery of central focus of lesion.

the enamel less susceptible to acid erosion. Gottlieb (21) apparently held a similar view of an accumulation of debris during the carious process but employs the term "enrichment."

In discussing the paper of Enright, Trescher and Friesell, Rosebury (22) pointed out that he was partially able to retain organic fragments in incompletely decalcified enamel. He was primarily concerned with the idea that artifacts might be produced through the employment of a fixing agent. He, too, was apparently unaware of the importance of fixation in the recovery of the organic matrix from the enamel structure. Frisbie, Nuckolls and Saunders (3)

made a similar observation associated with the development of the carious lesion when fixation of the entire structure was incomplete. The appearances found by these authors in stained enamel, when viewed in relationship to the full width of the enamel matrix, can be shown to be an early stage in matrix degradation, wherein the alteration of the matrix structure permits fixation at a more rapid rate than in the normal structure. When prepared for histological examination, the carious enamel matrix will exhibit a more intense tinctorial reaction than the unaffected surrounding tissue.

Weinmann, Wessinger and Reed (23) carried out a study on calcification of the organic enamel matrix. They employed a fixing agent which, by producing

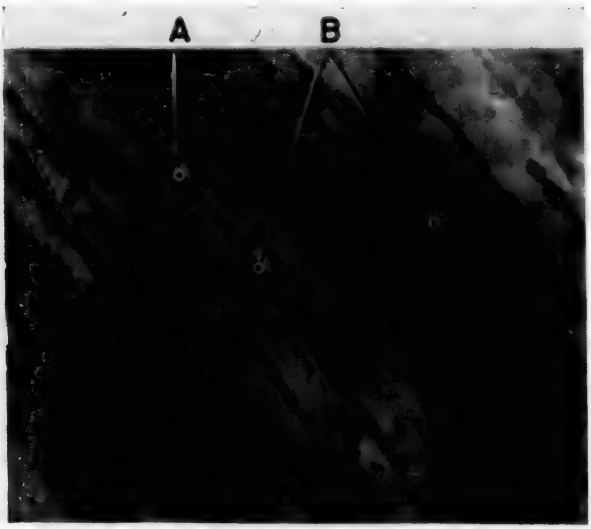

Fig. 13. Demineralized organic dentin matrix from adult human tooth. 4 μ neutral formol fixation. MacCallum's Gram × 1140. Note penetration of the inner walls of dentinal tubule. A. Dentinal tubule. B. Advanced penetration along inner wall of the tubule by Gram-positive spheroidal appearing microorganisms. Note: Morphological similarity between spearheading microorganisms in enamel and dentin.

a precipitation of the protein matrix in the area undergoing mineralization, tends to delay, if not prevent, complete fixation of the enamel structure. They concluded that after a mineral deposition in the matrix had been going on for some time, the mineralizing matrix became acid soluble. In contrast, the areas of lesser mineralization approximating the cervical line were found to be acid insoluble. In our opinion the different rates of penetration of the chemical agent in the more heavily mineralized areas when compared with areas of lesser mineralization, is responsible for this observation. The fixative agent penetrated the areas of heavy mineralization to a lesser degree, or not at all, and therefore resulted in an incomplete denaturation of the mineralized protein content of the enamel, the undenatured protein being more soluble than when

the penetration was more complete. Incomplete denaturation allowed these organic and inorganic constituents of the more densely mineralized part to go into solution at approximately the same time and the results obtained cannot, therefore, be ascribed to differences in acid solubility.

Saunders, Nuckolls and Frisbie (1) and Frisbie, Nuckolls and Saunders (3) stressed the importance of appropriate fixation and denaturation of the protein matrix if it were to be recovered in any quantities and to show structural detail from either the developing, fully erupted, or carious tooth. A study conducted in our laboratory on the action of fixative agents in gelatin (24) suggested that if distortion and artifact are to be minimized during fixation, the agent must produce a complete and continuous denaturation of the substance. Any chemical agent which tends to precipitate the organic structure will eventually produce a barrier to the further ingress of the fixative agent. As in previous investigations, gelatin experiments demonstrated that aqueous 10% neutral formol produced the least change in structure undergoing fixation.

The action of formol in producing protein denaturation renders the organic matrix of the enamel less soluble to the acids used in decalcification, permitting it to be recovered on removal of the calcium salts. It is admitted that the acids employed in decalcification no doubt cause some solution of the organic matrix along with the mineral complex. However, a sufficient amount of the matrix is retained to permit precise histological study and the establishment of its relationship to the pathological process of caries of the enamel. In addition, our studies lead us to believe that there is a greater ratio of organic material to the inorganic complex than has been heretofore determined. The advent and refinements of microtechnics may bring about a re-evaluation as to the ratio of organic to inorganic content of the enamel and the nature of the organic matrix itself. Such procedures are being investigated in our laboratory.

Frisbie, Nuckolls and Saunders (3) described several distinct types of extension of the carious lesion. Further studies extend and clarify these early observations. Our recent unpublished observations on caries in the hamster indicate that the plaque and the surface lesion may have their inception in the protein of the secondary cuticle which remains adherent to the protected surfaces of the teeth following eruption. An extension of this process results in the surface penetration of the uncalcified organic tracts and the establishment of carious lamellae. A similar process has long been observed and recognized with regard to the organic content in caries of pits and fissures in the human, the organic inclusions affording a favorable medium for bacterial attack which progresses to the deeper parts of the enamel and dentin structure.

With appropriate fixation and careful handling it is possible to retain the bacterial plaque in situ overlying the area of superficial carious enamel. Depending on the surface conditions, the plaque may vary in thickness from a visual accumulation of material to one of microscopic proportions which cannot be detected by instrumental examination. As in the deeper lesion, the morphological relationship of the organisms seems to be relatively constant. The thread forms predominate in the superficial layers, but the spheroid organisms

are found in immediate approximity to the surface zone of active enamel disintegration. From our preliminary studies of the plaque it seems permissible to state that its formation progresses in a rather stereotyped way. The authors have placed removable appliances in the oral cavity. These appliances contained plastic cover glasses which were removed, stained and studied under the microscope at regular intervals. The preliminary findings—which are admittedly incomplete—serve to throw some light on the development of the plaque. There is first an accumulation of an amorphous mucinous background in which the coccoid and filamentous organisms subsequently make their appearance. This observation would seem to concur in general with the appearance of bacteria within the plaque on the surface of the tooth. The relationship of the plaque to the surface of the tooth in early caries, which occur shortly after tooth eruption, is in all probability associated with the presence of the organic secondary cuticle. Observations with regard to animal experimentation remove these deductions from the realm of conjecture.

The matrix immediately underlying the surface plaque early exhibits an increasingly intense acidophilic reaction to stains when compared with the deeper placed and normal matrix. This acidophilic alteration is the earliest evidence of observed pathological change and may extend into the substance of the enamel for a considerable distance ahead of its solution.[6] Concomitant with, and closely following this change, is a progressive loss of structural detail in enamel elements; and when the pathological change is advanced, the matrix becomes almost homogenous. As yet the organisms may not have penetrated the cores of the rods and inter-rod intervals. With the establishment of the superficial carious lesion, further progress is observed to extend along narrow irregular tracts[7] or to spread out so as to include large surface areas.[8]

One form of caries is evidenced by narrow, irregular tracts which penetrate deeply and on superficial examination of routinely stained preparations, closely resemble the classically described enamel lamellae and may be mistaken for them in preparations in which the preservation of the enamel matrix is incomplete or imperfect. Under the higher power of the microscope and with bacteriological stains, these tracts are found to consist of from one to several isolated rods in thickness, the cores of the rods being packed with organisms. The infected rod undergoes mild degenerative changes which produce the differential staining reactions seen in routine preparations. In some instances the infection is confined to straight courses following what we consider to be incompletely mineralized rods to the dentinoenamel junction and there establishes a focus of *retrograde spread*. It has been suggested that the enamel rods may remain immature in development and mineralization and are, therefore, more susceptible to invasion by microorganisms. In the earlier stages of breakdown, it is impossible to determine the presence of these tracts by roentgenographic

[6] See reference 3, Superficial Caries.
[7] See reference 3, Deeply Penetrating Caries.
[8] See reference 3, Extensive Caries.

methods. Their course can be detected only by serial histological section. Since this tract widens with extension of the carious process, it may or may not become possible to determine its presence through the medium of the roentgen ray. A good example of a carious tract in the enamel involving the dentin is illustrated by Thoma, (25) "Fig. 615, Bite-wing film showing lamella in mandibular premolar." These advanced carious tracts should not be confused with uninfected lamellae which cannot be determined by the roentgen ray.

In another type of caries the invasion spreads along an irregular course involving rods and inter-rod intervals which implicate neighboring rods for some distance, thence down the core to infect deeper levels of the enamel. We regard these tracts as pseudo-lamellae or incompletely mineralized areas which may be associated with periods of nutritional deficiencies during development.

It has been demonstrated (1) that the dentino-enamel membrane is an apparently homogeneous matrix and that mineralization begins as soon as the structure is established. The mineral density of this layer rapidly increases up to the time of eruption until its density exceeds that of both the enamel and dentin. We believe that this extreme density of the dentino-enamel matrix is in part responsible for the retrograde spread on the enamel side before the dentin becomes infected. The recent observation of Keyes (26) on the carious lesion in the Syrian hamster indicates that the spread along the dentino-enamel junction agrees in a general way with our earlier observations in the human.

The morphological and physiological characteristics of the dentino-enamel junction would preclude the possibility of surface acids being drawn down the rod elements from the tooth surface toward the pulp.

A different appearance of the pathological process is associated with the production of extensive caries of the matrix[9] (so-called rampant caries). In this type parts of external surfaces or entire surfaces become involved and there is a development of multiple irregular channels of dissemination. This process has its inception as a multiple surface lesion, the pathological features of which are no different from those described under superficial caries. When the process is advanced, histological examination reveals an almost complete solution of the matrix. Upon visual examination the surface is chalky in appearance and may readily flake under the pressure of an instrument. In this stage the process leads to rapid and extensive superficial cavitation. We regard this type of caries as being established through a lesser order of calcification which permits a rapid penetration and destruction of the organic matrix by microorganisms and results in an early breakdown of the tooth surface.

The term *restricted caries* is applied, for convenience, to designate a different mode of penetration, although the pathological process is exactly the same as in superficial, deep and extensive caries. The surface enamel presents a brown-stained area. As observed in ground sections, the discoloration is found to extend to varying depths and, in some instances, involves the dentino-enamel junction. The stained areas are found chiefly in protected positions of the

[9] See reference 3, Extensive Caries.

tooth, such as within interproximal areas, where they cannot be readily observed. These stained surfaces are hard in the physical sense and cannot always be detected by present roentgenographic diagnostic methods. The lesion is slowly progressive and has been frequently designated in the literature as arrested caries. However, clinical observations have shown that when the lesion has been followed closely, it continues to increase in size and depth and ultimately involves the dentin. In the end it is followed by a physical disintegration of the surface. Depending on the structural development of the enamel and its mineralization patterns, the carious lesion may not necessarily extend to the dentino-enamel junction before a surface breakdown occurs. This lesion was recognized by W. D. Miller (27). He demonstrated this condition in ground sections and found that the surface of the enamel was hard and intact. The appearance was attributed to a destruction of the inorganic interprismatic substances by acids. According to Miller, the presence of the enamel cuticle[19] was responsible for the retardation of acid solution of the inorganic component, thereby resulting in an arresting of the carious process and protection of the tooth. This interpretation is understandable when one considers the hypothesis of J. Leon Williams (28) and others, who, by their early investigations, considered the enamel to be almost wholly inorganic in nature, and therefore could undergo degradation *only* through an acid decalcifying process. Any remnants of organic material which might be present in the enamel were considered to be of no consequence in the carious process. Therefore, acid must evolve from some source, and this was accounted for by the fermentation of carbohydrates on the surface of the tooth.

Our studies of human teeth clearly demonstrate that these intact, stained areas are carious in nature and slowly progressive. Deakins (29), investigating carious dentin, suggests that this brown stain is a melanin resulting from protein degradation. Gottlieb (21, 30, 31) has emphasized that a yellow pigmentation accompanies a proteolytic carious process. He suggests that the lesion is the result of bacterial invasion. However, since his investigations are largely concerned with ground sections, he has never histologically demonstrated the presence of a complete and continuous matrix and its invasion by microorganisms.

The actual pathology of restricted caries differs in no way from that observed in superficial and deeply penetrating or extensive caries. The presence of the lesion is associated with the appearance of a brown stain on the tooth surface and its advance into the deeper structures of the enamel is associated with a degradation of the protein matrix and the presence of microorganisms.

It has been previously pointed out that when the breakdown of the organic matrix is more advanced in restricted caries, there is a variation in morphological types of organisms. The apparently spheroidal forms are fewer in number

[19] It is presumed that Miller referred to the organic secondary cuticle.

We should like to express our thanks and indebtedness to Professor J. B. de C. M. Saunders for his continued interest in the problem, to Miss Barbara Killian for her technical supervision, to Dr. Nina Simmonds, Dr. Charles Craig and Dr. Benjamin Dienstein for their helpful suggestions, and to Mrs. H. Whitaker for secretarial assistance.

while the filamentous types are observed to predominate. This relationship to the central focus is relatively constant in this lesion. Thread forms, together with those which appear to be spheroidal in form, are found in greater numbers along the periphery of the lesion. The appearance of the thread forms in the central focus points to the possibility that these organisms have more than a casual relationship to the carious process. In the center of the focus and in areas where necrosis is almost complete, there are relatively few organisms. This characteristic positioning of the bacteria would tend to indicate that they are in greater abundance at locations where the uninfected matrix is first being attacked. The surface of the tooth is intact, and this activity on the part of the various types of organisms is confined within apparently hard enamel. The infection extends from the surface and may spread in all directions from within the enamel, in this way undermining the sound overlying tooth surface.

It is important to emphasize that if the organisms found in the deep focus were dependent on their nutrient being diffused in from the tooth surface, their relationship to the carious lesion would be inverse to their positions. It then follows that these organisms would tend to be found in greater abundance in the center of the lesion. However, the organisms operating in the lesion are found invariably at the periphery of the active lesion. If this process were one of acid solution resulting from carbohydrate degradation at the site of the organisms, an *undermining* lesion of this type could not have occurred. An acid solution of the inorganic constituent of the enamel could only appear as a cavity from its first inception.

When the lesion becomes advanced in extent, it eventually undergoes physical breakdown and cavitation results. In this broken surface all types of organisms are found to occupy the open cavity. It has been reported that a marked increase in the *Lactobacillus acidophilus* index accompanies cavitation (32). Cavitation, however, is the end result of the carious process, and the microorganisms operate for varying lengths of time before a break in the surface of the tooth occurs. We agree that the type of the lesion and the rapidity of development is in direct relation to the maturation of the matrix and degree of mineralization of the structure.

SUMMARY

The development of the carious lesion in enamel of the human has been followed from the earliest microscopic stages to the final cavitation of the surface. Several distinct types of penetration by microorganisms have been described. Another type of slowly progressing caries (restricted caries) has been studied. The gross appearance of the surface lesion is that of a brown-stained spot on the intact enamel surface of a tooth. The following observations were made.

1. In undecalcified ground sections the brown stain may extend to the dentinoenamel junction and may involve the dentin.

2. In this type of lesion the spherical microorganisms have long since penetrated the surface and have established a central focus of infection within the enamel.

3. A microscopic plaque overlies the stained carious surface of the tooth. The plaque consists of an amorphous background in which filamentous organisms form a dense mat and have a characteristic parallel arrangement to one another.

4. In the deeper part approximating the disintegrating tooth surface these filamentous forms are seen to project themselves directly into the surface lesion so as to appear partly within the degraded organic mass.

5. These organisms apparently make their appearance following the establishment of the superficial carious process. Morphologically the primary invaders have a spheroidal appearance.

6. The macroscopic appearance of the surface lesion is that of a hard, intact enamel surface. The microscopic observation is that the area of organic degradation may have had its initiation from multiple portals of entry on the tooth surface.

7. With deeper penetration of the infection, a central focus of infection is established and multiple irregular channels spearhead in all directions beneath an uninvolved surface so that islands of apparently uninfected matrix are isolated by the anastomosing of these channels. These invading organisms are of the spheroidal type.

8. Concomitantly with the spearheading tracts, the peripheral matrix of the central focus undergoes progressive disintegration, leaving a shell of uninvolved enamel on the surface of the tooth.

9. The infected organic matrix takes on a more intense acidophilic staining reaction when compared with the normal surrounding matrix. This increased staining reaction is the earliest indication of a pathological change in the matrix.

10. The increase in acceptance of the dye is closely followed by a progressive loss of structural detail; when the alteration is advanced, the matrix becomes almost homogenous in appearance.

11. The spheroidal appearing microorganisms are found in greater abundance at the perimeter of the central focus where the organic rods have begun to undergo a definite degradation and solution.

12. In the center of the focus where the breakdown of the organic rods is nearing completion, relatively few organisms are to be found.

13. The infection, having reached the dentino-enamel junction, then extends laterally along this structure on the enamel side, opening up the dentinal extremities of the rods and inter-rod intervals and thus establishes retrograde spread.

14. The lateral spread of the focus is at first confined to the enamel, and only when disintegration is advanced does the dentin become involved. This characteristic spread is due, in part, to the nature and mineralization of the dentino-enamel junction.

15. The first microorganisms to invade the dentin are of the same spheroidal type as those seen in the early invasion and spearheading of the enamel lesion.

16. The spheroidal forms first penetrate along the lateral walls of the dentinal tubule but are found in the center of the tubule only after the dentinal fibril has become affected.

17. The microorganisms associated with the carious lesion and the character of this lesion are essentially the same in both the enamel and dentin structures. The greater content of organic material in the dentin accounts for the greater rapidity of the breakdown.

18. When the degradation of the enamel matrix in the central focus is in its final stage, there is a variation in the morphological types of organisms present. The spheroid types no longer predominate, and threadlike forms are found in greater numbers.

19. The appearance of the thread forms in the central focus points to the possibility that these organisms may have more than a casual relationship to the carious process.

We believe that these observations of the carious process operating beneath apparently unbroken and intact enamel surfaces further advance our original hypothesis: that dental caries is fundamentally a degradation of the organic matrix of the enamel resulting from the enzymatic action of microorganisms, rather than a simple acid decalcification and removal of the inorganic salts.

REFERENCES TO LITERATURE

1. Saunders, J. B. de C. M., Nuckolls, J., and Frisbie, H. E., Amelogenesis, *J. Am. Col. Den.*, **9**: 107, 1942.
2. Nuckolls, J., Saunders, J. B. de C. M., and Frisbie, H. E., Amelogenesis, *J. Am. Col. Den.*, **10**: 241, 1943.
3. Frisbie, H. E., Nuckolls, J., and Saunders, J. B. de C. M., Distribution of the Organic Matrix of the Enamel in the Human Tooth and its Relation to the Histopathology of Caries, *J. Am. Col. Den.*, **11**: 243, 1944.
4. Frisbie, H. E., Further Studies on the Histopathology of Caries of the Enamel in the Human Tooth, (Abstract), *J. D. Res.*, **25**: 166, 1946.
5. Kossa, J. von, Uber die im Organismus kunstlich erzengbaren Verkolkungen, *Beitr. path. Anat.*, **29**: 163, 1901.
6. Frisbie, H. E., Caries of the Dentin, (Abstract) *J. D. Res.*, **24**: 195, 1945.
7. Baumgartner, E., Uber das Wesen der Zahnkaries mit besonderer Berucksichtigung der Histologie des gesunden und kariosen zahnschmelzes, *Dtsch. Monatsschr., F. Zahnheilk.*, **29**: 322, 1911.
8. Fleischmann, L., Zur Pathogenese der zahnkaries, *Ztschr. f. Stomatol.*, **19**: 153, 1921.
9. Malleson, H. C., The Histology of Enamel Caries, *Brit. D. J.*, **46**: 907, 1925.
10. Bodecker, C. F., The Bacterial Invasion of the Enamel in Dental Caries, (Preliminary Communication) *D. Cosmos*, **69**: 987, 1927.
11. Bodecker, C. F., Die Bacterien im Schmelzgeweba al Ursache karioser Vorgange, Sonderabdr. aus Viertel jahrsschrift f. Zahnheilk, Verlog von Hermann Meusser in Berlin, 1928. Heft 2.
12. Bodecker C. F. and Bodecker, H. W. C., The Bacterial Destruction of Dental Enamel, *J. D. Res.*, **9**: 37, 1929.
13. Bibby, B. G., The Organic Structure of Dental Enamel as a Passive Defense against Caries, *J. D. Res.*, **12**: 99, 1932.
14. Berke, J. D., Studies in the Histology and Pathology of Human Enamel, *D. Cosmos*, **78**: 700, 1936.
15. Berke, J. D., New Findings in the Histology and Pathology of Human Enamel, *J. A. D. A.*, **27**: 1229, 1940.
16. Beust, T. B., *Disc.* Bunting, R. W., and Palmerlee, Faith, The Role of Bacillus Acidophilus in Dental Caries, *J. A. D. A.*, **12**: 381, 1925.

17. Beust, T. B., Microorganisms and Caries, *J. A. D. A.*, **17**: 1536, 1930.
18. Enright, J. J., Friesell, H. E., and Trescher, M. O., Studies of the Cause and Nature of Dental Caries, *J. D. Res.*, **5**: 759, 1932.
19. Bibby, B. G. and Van Huysen, G., Changes on Enamel Surface, A Possible Defense Against Caries, *J. A. D. A.*, **20**: 828, 1933.
20. Bibby, B. G., Neglected Factors in the Study of Dental Caries, *J. A. D. A.*, **22**: 222, 1935.
21. Gottlieb, B., Dental Caries, *J. D. Res.*, **23**: 141, 1944.
22. Rosebury, T., *Disc.*, Ref. 18.
23. Weinman, J. P., Wessinger, J. D., and Reed, G., Correlation of Chemical and Histological Investigations on Developing Enamel, *J. D. Res.*, **21**: 171, 1942.
24. Nuckolls, J., Unpublished Data on the Nature of Various Fixative Agents on Blocks of Gelatin.
25. Thoma, K., Oral Pathology. Ed. 2, (St. Louis, Mosby: 1944), Fig. 615.
26. Keyes, P. H., Dental Caries in the Syrian Hamster, *J. D. Res.*, **25**: 341, 1946.
27. Miller, W. D., A Study of Certain Questions Relating to the Pathology of Teeth, Tr. 4th International Congress, **1**: 237, 1904.
28. Williams, J. L., On the Formation and Structure of Dental Enamel, *D. Cosmos*, **37**: 101, 269, 453, 1896.
29. Deakins, M., The Isolation of Melanin-like Substance from Carious Organic Matrix of Human Dentin, *J. D. Res.*, **20**: 39, 1941.
30. Gottlieb, B. and Hinds, E. C., Some New Aspects in Pathology of Dental Caries, (Abstract) *J. D. Res.*, **21**: 317, 1942.
31. Gottlieb, B., Two Types of Undermining Dentin Caries, *J. D. Res.*, **12**: 73, 1945.
32. Hemmans, E. S., Blaney, J. R., Bradel, S. F., and Harrison, R. W., The Microbic Flora of the Dental Plaque in Relation to the Beginning of Caries, *J. D. Res.* **25**: 195, 1946.

INVESTIGATIONS ON THE SALIVARY PHOSPHATES AND PHOSPHATASES[1]

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INTRODUCTION

Caries, periodontosis and disturbances of ossification, once considered primarily problems of calcium metabolism, are now recognized as associated with phosphorus metabolism through the calcium-phosphorus balance. The results of salivary phosphorus analyses, which offer a key to the phosphorus metabolism, have not been uniform. A critical review by Becks and Wainwright (5) show inorganic phosphorus values reported between the extremes of 2.5 and 70 mg. % with normal values between 10 and 24 mg. %. The reported range for total phosphorus lay between 4 and 22 mg. % and normal values were given as 14 to 18 mg. %. The normal values for total acid-soluble phosphorus were given as 10 to 24 mg. %. Such wide variations in normal values would be considered inaccurate in other body secretions and suggest the need for critical review of previous salivary analyses and for further study.

Consideration of the age and general health of the subjects and the time and manner of collection of the saliva specimen is important. The importance of these factors and of the salivary phosphatases is emphasized in this study. Since saliva contains phosphatase substrate (organic phosphorus esters) and 3 phosphate splitting enzymes of comparatively greater activity than those of milk and urine, the mechanism regulating the phosphorus level must be considered.

It is probable that normal and pathologic values cannot be expressed as given mg. % but rather in terms of ratios of the different phosphorus fractions. It seems possible to raise the inorganic salivary phosphorus level by increased intake of phosphorus-containing foods but impossible to raise the organic salivary (or blood) phosphorus level by any voluntary procedure. The synthesis of organic phosphates of the blood and saliva is an important unsolved problem (9).

REVIEW OF LITERATURE

Very few of the reported salivary phosphorus analyses are of value for comparative or statistical study. Most report inorganic phosphorus and calcium with an occasional author giving organic phosphorus values.

Youngburg (28) and Mathis (18) found total salivary organic phosphorus to represent 4 to 5% of the total salivary phosphorus while Axmacher (2) found 13% and Price (20) 50% organic phosphorus. For the organic acid-soluble phosphorus fraction Youngburg (28) reported 0%, Mathis (18) 4.5%, Smith (23) 9%, Adamson (1) 17% and Weber (27) 59%. This portion probably

[1] Received for publication October 1, 1946.

includes the hexosemonophosphates, diphosphoglyceric acid, creatinephosphate and adenylepyrophosphate. Organic acid-insoluble phosphorus, which includes salivary phosphorus from mucin, from nucleoprotein of the epithelial cells and the phospholipoides, was estimated at 0% by Mathis (18) and at 14% of the total acid soluble phosphorus by Entin and Geikin (12).

There may be many reasons for these wide variations including the use or disuse of the centrifuge, defective technic and variation in the phosphorus content of the epithelial cells. It may also be suggested that variations of the organic phosphorus values of the saliva actually are greater than those for inorganic phosphorus influenced by unevaluated factors such as cellular oxidation.

Literature on salivary phosphatases is scanty. Demuth (6), who used hexosediphosphate as his substrate, appears to have been the first to describe a salivary phosphatase. Adamson (1) and Smith (23) examined oral phosphatases in relation to salivary calculus formation. They concluded that the enzyme which can be liberated from traumatized gingival tissue is capable of of hydrolyzing the organic phosphoric esters of the blood and saliva. Adamson gave pH 9 and Smith gave pH 5.5 as optimum for salivary phosphatase. Giri (13) identified salivary phosphatase with urine phosphatase. Glock, Murray and Pincus (14) called attention to the microorganisms of saliva as possible sources of salivary phosphatase. Zander (29) and Valloton (25) have demonstrated the presence of phosphatase in the sub-gingival tissues where it may be liberated by inflammatory reaction. Lura (8) examined the effect of storage and pointed out the synthesizing effect of salivary phosphatase.

EXPERIMENTAL

Materials and Technique

Previous investigators rarely paid attention to the origin of the saliva or to the other facts which may have particular interest. Most frequently the author's own saliva or that of his assistants was used for the analyses, and it was seldom indicated whether the saliva originated from old or young individuals, from caries-free or caries susceptible individuals, nor was the exact time for collecting saliva indicated. In the following experiments, an effort was made to avoid these oversights by using saliva from healthy children and adults (ages 5 to 25 years), who had been observed through very long periods. Most of these patients were from the author's own practice. Some were children from the state reformatory, where the frequency of caries is very low and where the children are living under a very healthy and regular dietary condition.

Before the special experiments were carried out some qualitative salivary analyses of all the caries-free children were performed. These included: 1) The flow and quantity of saliva, 2) pH buffer quality of saliva, 3) viscosity, 4) sugar analysis, 5) lactic acid analyses, 6) ammonia analyses, 7) bacterial analysis by growth on pepton agar of pH 7.2 and on tomato agar pH 5.5 (11). If the salivary samples showed great deviations, especially in the pH values, viscosity, or lactic acid content, or more particularly in the bacterial content,

the samples were rejected so that the rest of the experimental material furnished as uniform conditions as possible. The author never used stimulated saliva but only resting saliva obtained at least 2 hours after the last previous meal. In many instances, morning or fasting saliva obtained before the first meal was used, or in other cases, the saliva was collected before lunch at 10 o'clock. The quantity obtained generally amounted to 5 to 8 ccm. collected over 10 to 15 minute period. Unless otherwise noted unfiltered and uncentrifuged saliva was used for all the described experiments.

Some authors, including Becks (3) have recommended centrifuging because this is required for the calcium analyses. However, when the phosphorus analyses are to be studied, the centrifuge only offers slight advantage. The contaminations which can be removed by the centrifuge are rather insignificant, provided pure, clean and normal salivary types are selected. By centrifuging we risk the loss of a great part of the organic phosphorus fractions which play a leading role in the present work. The main purpose of the analyses is to get as complete a picture as possible of the most important components and enzymatic qualities of the saliva, and any filtering, centrifuging or removing of the albumens would only eliminate the most characteristic compounds or disturb the enzyme relationships. When the removal of these components was essential as in investigating the origin of certain enzymes or the effect of purified saliva, a glass filter Shott 3 G on 5 with a vacuum pump was used. The effect of bacteria was eliminated by adding some drops of toluene (1%) or chloroform to the saliva.

Salivary samples were taken from persons of both sexes. As no difference in the enzyme content or in the phosphate values could be demonstrated between the sexes, they were not separately classified in the tabulations.

For the determination of the total phosphorus content the so-called 'wet' or 'dry' standard ashing-procedure of the whole untreated saliva can be used. The total acid-soluble phosphorus requires a previous removal of the albumens by trichloracetic acid. The 'wet' washing procedure is the most convenient and was carried out in all the following experiments.

Technique for the 'wet' ashing procedure. The reagents are: a) Sulphuric acid solution (30 ccm. concentrated sulphuric acid diluted with water to 100 ccm.), b) Merk's perhydrol (peroxyde of hydrogen 35%), C) Acid-free molybdate reagent (5% ammonium-molybdate).

To 1 ccm. of saliva pipetted into a micro-kjeldahl flask, 1 ccm. of sulphuric acid solution is added. This is heated over the Kjeldahl-burner with little flame until sulphuric vapours are liberated. The black, carbonated liquid is then cooled. A drop of perhydrol is added, and the flask is heated again until the liquid remains clear and new sulphuric vapours appear. After cooling, 2 ccm. of acid-free molybdate reagent, 1 ccm. of sodium sulphite 25% and 1 ccm. hydroquinone solution (0.5%) are added and diluted to 10 ccm. with water.

After 60 minutes, colorimetry of Hellige-Universal-Colorimeter is performed by comparison with the standard solution treated exactly as in the test (0.3296 grs. of KH_2PO_4 diluted in 250 ccm. of water. 1 ccm. = 0.3 mg.P.).

The calculation of the inorganic phosphorus is done by means of an adjusting curve constructed on the basis of some known standard solution.

Determination of inorganic phosphorus. One ccm. of the material is diluted with water to 5 ccm., 1 ccm. of trichloracetic acid (20%) is added and the liquid is filtered. To 5 ccm. of filtrate is added: 1) 2 ccm. of acid-containing molybdate reagent (10 gr. pure ammonium-molybdate are diluted to 100 ccm. hot aqua dist. This solution is slowly added to a cooled mixture of 30 ccm. of concentrated sulphuric acid in 50 ccm. of aqua dist). 2) 1 ccm. of 20% sodium sulphite. 3) 1 ccm. hydroquinone (0.5%) + 1 ccm. of water. After 60 minutes comparison in the colorimeter is made against a standard treated in the same manner.

Calculation

Five ccm. of the deproteinated filtrate corresponding to 25/6 before adding trichloracetic acid from which the content of the original material is calculated (multiplication by 1.2). It is important that the constant reading of the colorimeter always takes place exactly 60 minutes after the adding of the reagents, because further delay would change the color values.

Calculation of organic fractions. Total organic P = Total P+ inorganic P, organic non-acid soluble P = Total P + total acid soluble P, organic acid soluble P = Total acid soluble P + inorganic P. *Statistical.* The biometric valuation of the material included the following values: a) the arithmetic mean (M), b) the standard deviation, (6), c) the standard of the mean (σ_M) (26). The error of the above mentioned procedure does not exceed ± 2% within the range of amounts of inorganic phosphorus, which can be considered in this work, that is, from 0.007 to 0.035 mg. P.

DATA

The Phosphates of Saliva

A. *Ranges and average values of total phosphorus content of saliva.* In order to show possible diurnal shifts in the total phosphorus content of saliva, some experiments were performed in which salivary tests from the same persons were taken at different hours, usually in the morning before the first meal (8 o'clock) and afternoon (2 hours after dinner) (Tables I and II).

The observed diurnal variations of the total phosphorus content of saliva can be explained by: 1) diurnal variations of the content of bacteria and epithelial cells, 2) influence of food intake. To demonstrate the possible variations caused by the bacteria, leucocytes and epithelial cells of saliva, some analyses were performed with sterile saliva (boiled and filtered) against the non-sterile (Table III). As the boiled and filtered saliva also shows diurnal variations, it is obvious that these cannot exclusively be due to the bacteria and contaminations, but the variations must be influenced by diet, adsorption of phosphorus and other internal causes.

B. *Ranges and average values of inorganic phosphorus content of saliva.* (Tables IV, V and VB).

C. *Ranges and average values of total organic phosphorus of saliva.* (Table VI). Greatest individual variations are dominant in this fraction. Compared with the results of previous investigators the present values seem to be much

TABLE I

Random values from 120 analyses on 120 persons of both sexes. Age 5–25 years. All caries-free. Resting saliva collected between 8 and 10 A.M.

CASE NO.	AGE	TOTAL P., MG%	CASE NO.	AGE	TOTAL P., MG%
6	8 years	17.2	118	16	18.1
64	14 years	19.4	75	17	32.4
8	8 years	21.7	107	25	22.2
28	12 years	16.4	81	11	14.8
10	10 years	19.8	50	12	23.4
77	24 years	28.9	98	18	26.3
13	10 years	22.2	44	6	15.5
9	18 years	23.8	28	12	16.8
16	7 years	16.4	75	16	21.4
31	19 years	17.4	92	12	19.2

Mean (M) = 20.4
Standard deviation (σ) = 4.2
Error of the mean (σ_M) = 0.38

TABLE II

Random values of 85 analyses

CASE NO.	TOTAL P, MG. %	
	MORNING VALUE	AFTERNOON VALUE
6	18.5	17.3
19	21.1	20.2
4	22.5	22.9
21	19.8	17.9
3	16.7	17.4

TABLE III

Phosphorus content of sterilized and unsterilized salivas (Average of 30 different individuals)

	TOTAL P, MG. %	
	MORNING	EVENING
Ordinary, unsterile saliva.....	20.2	18.8
Boiled and filtered saliva.....	17.6	16.4

higher (20 to 25% of the total phosphorus). Seasonable variations are to be observed in this fraction.

D. *Ranges and average values of organic acid-soluble phosphorus of saliva.* To 2.5 ccm. of saliva 0.5 ccm. trichloracetic acid (20%) was added. After 15

minutes of filtering, 1 ccm. was used for analysis as described under the 'wet' ashing procedure. Correction for the trichloracetic acid is necessary (multiplication by 1.2). This fraction shows especially great individual variations. Some studies were carried out with saliva from individuals who had been ex-

TABLE IV

Random values from 180 analyses on 180 persons of both sexes. Age 5–25 years all caries-free. Resting saliva, collected from 8 to 10 A.M.

CASE NO.	AGE	P, MG%	CASE NO.	AGE	P, MG%
5	12	14.1	89	20	16.8
8	8	16.6	16	7	15.8
61	21	7.4	141	15	8.9
28	12	13.8	10	10	17.1
9	18	21.1	113	11	8.4
121	7	9.8	110	17	14.9
95	9	12.3	8	6	16.8
34	15	18.4	159	22	15.0
13	16	11.8	162	18	13.4
75	15	17.3	50	25	11.2

Mean (M) = 14.9
Standard deviation (σ) = 3.4
Error of the mean (σ_M) = 0.25

TABLE V

Diurnal variations of inorganic phosphorus of saliva. (Random values of 85 cases)

CASE NO.	INORG. P, MG%	
	MORNING	EVENING
8	17.8	16.2
12	14.4	13.1
7	13.6	14.8
23	8.9	9.2
14	15.9	15.8

TABLE VB

Diurnal variations of sterile and unsterile saliva. (Average values of 35 cases)

	INORG. P, MG. %	
	MORNING	EVENING
Unsterile saliva	15.1	14.2
Boiled, filtered saliva	14.3	13.6

posed to great physical fatigue, and the values were generally diminished under these circumstances. (Table VII)

E. *Ranges and average values of organic acid-insoluble phosphorus.* The investigations included the same subjects used in other portions of this study

but only 12 experiments gave positive values. In the rest of the material the values were so insignificant that they did not surpass the standard error.

Investigations on the acid-hydrolyzable phosphorus of saliva. To 5 ccm. of trichloracetic filtrate of saliva 1 ccm. of the same sulphuric acid solution

TABLE VI

Total organic phosphorus of saliva. (Random values of 50 cases)

NO.	YEARS	TOTAL P, MG. %	INORG. P, MG. %	TOTAL ORGANIC	% OF TOTAL
4	8	18.9	16.1	2.8	14.7
7	12	13.8	12.9	0.9	6.4
10	10	19.8	17.1	2.7	13.6
25	41	22.6	15.5	7.1	31.4
9	16	23.8	21.1	2.7	11.3
33	26	25.8	11.2	14.6	56.6
42	35	18.9	13.2	5.7	30.1
17	10	22.5	19.3	3.2	14.2
8	6	21.7	16.6	5.1	23.0
28	12	16.4	13.8	2.6	16.1
19	17	22.1	20.7	1.4	6.3
13	10	22.2	11.8	10.4	46.8
Mean		20.4	14.9	(M) = 5.5	20–25%
Standard deviation				(σ) = 3.9	
Error of the mean				(σ_M) = 0.55	

TABLE VII

Organic acid-soluble phosphorus of saliva. (Extremes of 30 cases.)

CASE NO.	AGE	A TOTAL ACID-SOL. P MG.%	B INORG. P MG.%	A-B TOTAL ACID-SOL. ORG. P MG.%	PERCENT OF TOTAL
35	16	14.9	11.2	3.7	18
8	8	20.2	16.6	3.6	16.5
19	17	22.0	13.8	8.3	37.6
15	16	27.3	17.1	10.2	40.0
18	8	20.4	16.6	3.8	13.6
42	35	18.3	13.2	5.1	28.5
7	12	13.5	12.9	0.6	4.4
5	12	16.6	14.1	2.5	15.0
Mean		19.8	14.9	M = 4.9	18–24%
Standard deviation				σ = 3.1	
Error of mean				σ_M = 0.56	

which was used for the determination of total P was added. One sample was heated 10 minutes and another 60 minutes by boiling on the waterbath. After cooling, analysis for inorganic P. as described above was carried out correcting for the acid addition as indicated in the calculation. Another sample was not heated and the difference between the sample heated for 10 minutes and that

not heated gives the quantity of the easily acid-hydrolyzable phosphate (Table IX). The 60 minute value is used to express the splitting of the not easily hydrolyzable compound (Table X). The saliva which does not show any increase by the short boiling with acid, very often shows an increase by the long boiling, so that it can be concluded that at least 2 well separated fractions are present.

TABLE VIII

Organic acid insoluble phosphorus. (Extreme values of 12 individuals.)

CASE NO.	AGE	TOTAL P, MG%	TOTAL ACID-SOL. P, MG%	ORGAN. ACID INSOL. P, MG. %	PERCENT OF TOTAL
35	16 yrs.	15.8	14.9	0.9	4.2
9	18 yrs.	23.8	23.6	0.2	0.8
42	35 yrs.	18.9	18.3	0.6	3.1
8	6 yrs.	21.7	20.2	1.5	6.1
19	17 yrs.	22.1	22.0	0.1	0.4
4	8 yrs.	18.9	17.9	1.0	5.3
Mean		20.4	19.8	M = 0.6	ca. 3%

TABLE IX

Acid-hydrolysis of trichloracetic acid filtrate of fresh morning saliva. (10 minute boiling).

CASE	INORGAN. P BEFORE HYDROL. MG%	INORG. P AFTER HYDROL. MG%	INCREASE MG%
1	17.5	21.2	3.7
2	10.3	14.6	4.3
3	9.7	12.0	2.3
4	16.8	18.2	1.4
5	15.1	17.7	2.6
6	18.4	19.4	1.0
7	14.2	16.9	2.7
8	10.8	13.9	3.1

TABLE X

Acid-hydrolysis of trichloracetic acid filtrate of saliva. (60 minute boiling).

CASE	INORG. P BEFORE HYDROL. MG%	INORG. P AFTER HYDROL. MG%	INCREASE MG%
1	17.5	23.6	6.1
2	10.3	16.0	5.7
3	9.7	16.3	6.6
4	16.8	21.6	4.8
5	15.1	19.3	4.2
6	18.4	24.2	5.8
7	14.2	21.3	7.1
8	10.8	14.3	3.9

Determination of the nature of the organic phosphorus in saliva.

The presence of complex, not easily hydrolyzed phosphates in the saliva, is demonstrated by the long hydrolysis with diluted acids and by determination of the reducing power before and after the hydrolysis by Hagedorn-Jensen's method for the estimation of blood sugars. While boiling is increasing free phosphate, reducing hexose-sugars are being disengaged from the compound (Table XI). Different possibilities may produce this increase of sugar. These include: 1) Augmentation of sugar from starch-containing ptyalin-splitting

TABLE XI

1 ccm. saliva hydrolyzed 30 min. with 0.1 n—H_2SO_4

	SUGAR MG%	PHOSPHATE MG%
Before boiling	12	16.1
After boiling	30	17.9
Increase	28	1.8

TABLE XII

1 ccm. protein free filtrate of saliva hydrolyzed 30 min.

	SUGAR MG%	PHOSPHATE MG%
Before boiling	5	16.4
After boiling	18	18.7
Increase	13	2.3

TABLE XIII

1 ccm. fresh saliva and 1 ccm. boiled saliva (3 min.)
Incubated 24 hours by 37. Toluene added

	SUGAR MG.%	PHOSPHATE MG.%
(a) Saliva before incubation	4.5	15.1
Boiled saliva before incubation	5.1	15.4
(b) Saliva after incubation	26.0	18.4
Boiled saliva after incubation	5.4	15.4
Increase	21.5	3.3

food remnants. For this reason it is necessary to wait some hours after the last meal and after the mouth has been cleaned before collecting the salivary sample. As the activity of ptyalin is very prompt, sugar from this source can easily be observed by a control test before the hydrolysis. 2) A possible hydrolysis of the glucoprotein mucin. By control with protein-free filtrate this possibility is excluded (Table XII). 3) Enzymatic hydrolysis of the hexose-phosphate caused by the salivary phosphatases. By incubation of freshly boiled saliva this possibility can be controlled (Table XIII). Also in

this case an increase of the reducing power is found in the enzyme-containing saliva, while the inactivated saliva only shows a very insignificant increase. In all these experiments Hagedorn-Jensen's estimation of blood sugars was used on quantities of saliva from 0.1 to 0.2 ccm.

Investigations on the salivary phosphatases

Methods. In the following experiments the estimation of phosphatase was performed by two different methods:

Substrate-mixture

1) Glycine or borate-buffer	pH 9.4	320 ccm.
2) n/10 NaOH		180 ccm.
3) 1% Disodiumbetaglycerophosphate		500 ccm.
4) 2% Mg-sulphate		10 ccm.

A. Briggs modification of Bell-Doisy's procedure was used. When a substrate-mixture with a lower pH, that is, 5.5, is required, an acetate-buffer is used instead of the glycine-sodium hydroxyed buffer.

The reagents for determination of inorganic P. are the same as described above.

Technique. The test-tubes are filled with 4 ccm. substrate mixture plus 1 ccm. fresh saliva. One tube is taken out immediately for determination of inorganic phosphate. The calculation is done by means of an adjusting-curve constructed on the basis of some known standard solution. The other test tubes are placed in the thermostat at 37° C. and determinations for inorganic phosphorus are performed at intervals. In some of the tubes boiled saliva is used as a control. It is important that the phosphorus content in the standard solution and the test solution be as near each other as possible, and that the difference does not exceed 50%. Furthermore, it is necessary that the hydrolysis of the substrate does not exceed 10%.

The ideal phosphatase reaction would be that in which at equal time intervals equal quantities of inorganic phosphorus were split off, but in reality the reaction curve is not a straight one but one which falls slightly because of the destruction of the enzyme by the alkaline reaction and because of the hydrolysis is checked by the increasing amount of inorganic phosphorus. For this reason the determination of initial hydrolysis will always be the best indicator of the amount of phosphatase, provided that the activity of the enzyme is not too weak.

It has been shown by several investigators (cited by Stein [24]) that the optimal concentration is 0.5% to 1% for the alkaline phosphatase and a little higher (1% to 2%) for the acid. Twenty hours incubation generally is used for the reaction time in dairy control work and the same reaction time was used in a previous work by the present writer. In the present study an effort was made to examine this matter more carefully.

Determination of the length of the reaction time of the salivary phosphatases. Technique. One ccm. of enzyme solution in different active percentages is

added to 4 ccm. sodium-glycerophosphate (0.5%). One portion of the enzyme-mixture is inactivated by boiling for 3 min. The *following* mixtures are made:

Pure active saliva = 100% enzyme
4 portion active + 1 portion inactivated = 80% enzyme
1 portion active + 1 portion inactivated = 50% enzyme
1 portion active + 4 portion inactivated = 20% enzyme
Inactivated saliva = 0% enzyme
4 ccm water + 1 ccm saliva = enzyme control
4 ccm substrate + 1 ccm water = substrate control
4 ccm of 0.5% sodium-glycerophosphate correspond to 2.88 mg P from which the percentage of the hydrolysis is calculated.

The results at various reaction times is shown in Tables XIV and XV. In the following experiments a reaction time of 3 hours was chosen. Some authors consider a phosphatase unit as the amount of enzyme which is required for setting free 1 mg. of inorganic phosphorous during 1 hour, but this circumscrip-

TABLE XIV

Effect of time of incubation on phosphorus components at pH 9.2 and Temperature of 37 degrees C.

ENZYME	P, MG.				
	0 HOURS	3 HOURS	6 HOURS	9 HOURS	12 HOURS
100 %	0.147	0.171 (+0.024)	0.192 (+0.045)	0.212 (+0.065)	0.228 (+0.081)
80 %	0.147	0.168 (+0.021)	0.183 (+0.036)	0.205 (+0.058)	0.213 (+0.066)
50 %	0.147	0.160 (+0.014)	0.171 (+0.025)	0.183 (+0.037)	0.188 (+0.042)
20 %	0.147	0.149 (+0.004)	0.151 (+0.006)	0.154 (+0.009)	0.155 (+0.010)
0 %	0.147	0.147	0.147	0.147	0.147
sal. cont.	0.140	0.142 (+0.002)	0.145 (+0.005)	0.147 (+0.007)	0.149 (+0.009)
sub. cont	0.007	0.007	0.007	0.007	0.007

tion has not purpose in the present work, where it is more important to compare the different values of phosphatase calculated from 1 ccm. of saliva.

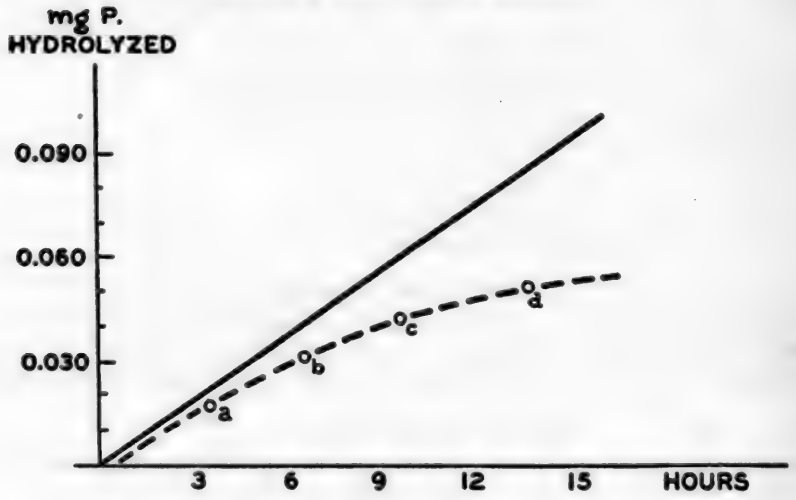
In the following experiments the phosphatase activity is considered as the amount of inorganic phosphorus in mg. % which is set free from 1 ccm. of saliva during 3 hours with sodium-glycerophosphate as a substrate.

B. In the second method di-sodium-phenylphosphate is used as substrate and the amount of free phenol is used as a measure of activity of the enzyme (21). The phenol reagent is 2.6 dibromkinonchlorimid with a solution of alkaline lead-acetate (for deproteination) at pH 9.2[1] (Table XVI). As standard color, 100 mg. crystalline phenol is diluted in 1 liter of distilled water. From this standard solution the following quantities are pipetted into 100 ccm. of distilled water: 0.5 ccm., 1 ccm., 2 ccm., 4 ccm. To 5 ccm. of each solution 2 drops of phenol-reagent are added, representing respectively 2.5, 5, 7.5 and 10

[1] The reagents can be obtained in tablets ready for use (Manufacturers "Ewos" Sodertalje, Sweden), as they are employed in the dairy industry for milk control.

TABLE XV

Typical progress of the salivary phosphatase reaction. With indication of the hydrolysis in mg. P at different hours. The straight line in the curve indicates the hydrolysis of equal P mg. per time-units



Percentage of theoretical hydrolysis.
a = 0.8%, b = 1.6%, c = 2.3%, d = 3%
(4 ccm substrate = 2.88 mg P.)

TABLE XVI

Samples of individual variations in the activity of the salivary phosphatases

Morning saliva. Incubation 3 hours at 37°

EXTREME VALUES 85	pH 5.5 MG%	pH 9.4 MG%	SHARER UNITS
Case no.			
4	3.1	2.0	15.0
2	1.6	1.2	10.0
10	0.8	2.9	17.5
15	2.0	1.7	12.5
19	7.1	3.6	25.0
8	2.9	0.6	5.0
22	4.5	2.5	17.5
21	2.7	1.5	12.5
30	3.4	2.8	20.0
14	4.4	3.1	22.5
6	3.5	2.4	17.5
31	5.4	2.7	20.0
9	4.0	2.1	15.0
12	1.6	0.8	10.0
18	6.0	3.1	22.5
Average of 135 cases = 4.3		2.4	15–17.5
Standard deviation σ = 1.62		1.55	
Error of the mean σ_M = 0.27		0.26	

units.[1] The above mentioned splittings generally correspond to 0.5 to 1.5% of the theoretical hydrolysis by 3 hours incubation.

Investigations on the range of quantity and origin of the salivary phosphatases

No indication of the range of the quantity of the salivary phosphatases in relation to other phosphatases of the body is given in the literature. Those authors who attempted to find the quantitative distribution of the phosphatases in the different tissues and liquids of the body, clearly point out the difficulties of this problem (15, 17).

The extraction and cleaning of phosphatases is not accomplished easily, whether they be from bone, liver, kidney, intestine, blood or saliva, because the enzyme is not equally distributed in the different cellular tissues.

Technique. The material was collected in fresh condition, for instance pieces of bone from the alveolar process, gingival tissue from small surgical operations, and teeth from extractions, and washed in distilled water and weighed. Afterwards the tissues were crushed with sterile tongs and an extraction solution composed of 1 part chloroform and 3 parts buffer solution was added in a quantity double the weight of the material. It was left 24 to 48 hours at room temperature. The extract was then centrifuged and pipetted off for estimation of the activity. Liquids such as milk, saliva and blood serum were used undiluted. The simplest determination of the activity is done by the Sharer method. If the material is very active, it must be diluted in order to bring the content down to measurable proportions, or the reaction time must be shortened proportionally and then calculated and compared. To obtain material for orientation an experiment was performed with enzyme extracts from pigs (Table XVII). The pig had not previously been used for salivary phosphatase experiments, but seems suitable for the purpose. As it appears from the above experiment the range of size of the salivary phosphatases seems to approach that of urine and milk, while the alkaline phosphatase of the blood is stronger than the salivary phosphatase. The acid phosphatase proportions of the blood and the saliva seem to be opposite.

The physiological range of size of the salivary phosphatases. The phosphatase activity of the different tissues depends to a great extent on the pH adjustment. The phosphatase activity of the blood is most marked by alkaline reaction, while that of saliva and the urine is most marked in acid reaction. (Tables XVIII and XIX).

The origin of the salivary phosphatases. Tables XX and XXI indicate the sites of origin of salivary phosphatase. The activity of the alkaline phosphatase is almost uniformly distributed in all fractions, while probably on account of the micro-organisms, the strongest effect of the acid phosphatase appears in the sediment.

Of all the oral tissues examined, the sublingual tissue seem to present the strongest phosphatase activity both in the acid and alkaline fractions. The presence of a possible increase of the salivary phosphatase may be due to the following 2 reasons:[1] A diffusion from the surrounding tissues through the

TABLE XVII

Salivary phosphatase of the pig as determined on 4 ccm. substrate + 1 ccm. enzyme solution incubated 3 hours at 37°

MATERIAL	pH 9.4 MG%	APPROXIMATE RANGE OF SIZE	pH 5.5 MG%	APPROXIMATE RANGE OF SIZE
Young bone	41.6	20	16.2	5
Kidney	32.4	12	25.2	10
Gingiva	14.4	7	12.4	7
Lungs	13.6	7		
Intestine	12.6	6	8.1	3
Liver	8.1	4		
Blood	5.5	3	11.8	0.7
Urine	2.1	0.8	3.1	1
Saliva	*2.3*	*1.0*	*3.2*	*1*
Pancreas	1.6	0.5		
Parotis	0.8	0.3	0.5	
Gall	0.3	0.2	0.2	
Milk	2.8	1	1.2	0.5
Dentin	1.0	0.5	0.0	0
Enamel	0.3	0		

TABLE XVIII

Quantities of phosphatase in material from different patients from the author's practice. (4 ccm. substrate + 1 ccm. enzyme, 3 hours incubation. 37')

MATERIAL	pH 5.5 MG%	pH 9.4 MG%	APPROXIMATE RANGE OF SIZE AGAINST SALIVA	
			pH 5.5	pH 9.4
Bone tissues	11.0	28.6	4	8
Gingival tissues	17.2	18.4	5	6
Periodontal membrane	7.0	16.2	2	6
Pulverized enamel	0	0.3	0	
Pulverized dentin	0	1.0	0	0.5
Tooth plaques	5.8	3.2	1.5	1.2
Mother's milk	1.2	4.8	0.5	1.8
Blood-serum	2.0	6.1	0.8	3
Urine	4.3	1.0	1.2	0.5
Saliva	3.2	2.4	1	1

TABLE XIX

Phosphatase content of tissues express in Sharer units. (Same material as used for determinations in Table XVIII.)

(5 ccm. "Ewos" substrate + 0.5 ccm. enzyme—solution. Inc. 1 hour 37'.)

Alveolar Process	60–70
Gingival tissues	70–80
Periodontal membrane	70–75
Enamel	0– 2.5
Dentin	10–12.5
Tooth plaques	30–40
Mother's milk	15–17.5
Blood serum	20–22.5
Urine	10–12.5
Saliva	12.5–15.

salivary glands and the blood vessels, 2) The quantity of phosphatase may be due to the bacteria and the micro-organisms of the saliva, from which it is disengaged by autolysis.

Regardless of the individual variations of the activity of the salivary phosphatases, it is necessary to reckon with constant activity, and no type of saliva is quite free from the enzyme.

The optimal pH of the salivary phosphatases. As there appears to be some disagreement concerning the optimal pH of the salivary phosphatases among the previous investigators, this question was investigated (Table XXII). Acid

TABLE XX

Sites of origin of phosphatase with fractionated secretion

(0.5 ccm. enzyme material + 5 ccm. "Ewos" substrate)

MATERIAL	SHAKER UNITS
Whole mixed saliva	12.5–15
Parotid saliva	7.5–10
Submaxillary	12.5–15
Centrifuged saliva	7.5–10
Sediment from centrifuge	15 –20
Filtrate	2.5– 7.5
Boiled saliva (control)	0.0

TABLE XXI

Sites of origin of salivary phosphatase

1 ccm. enzyme material + 4 ccm. substrate (glycerophosphate). Inc. 3 hrs. 37°

MATERIAL	pH 9.4 mg%	pH 5.5 mg%
Whole mixed saliva	2.3	3.6
Parotid saliva	1.4	2.5
Submaxillary	2.1	2.8
Centrifugate	1.8	1.6
Sediment	2.6	4.8
Filtrate	1.0	1.1
Boiled saliva (control)	0	0

saliva seems to have a greater hydrolyzing power than alkaline, and also seems to show fewer individual variations than the alkaline. Storage of saliva does not lower the effect during the first 3 or 4 days. After this period the activity slowly decreases. Addition of toluene to the saliva displaces the reaction to the acid side but does not reduce the hydrolyzing activity.

By addition of different buffer solutions to the saliva, the optimal pH appears on the acid side (pH 5.5) for almost all types of saliva (Table XXIII). This does not mean that the saliva contains only the acid phosphatase (the urine phosphatase) but rather that this phosphatase, by addition of the buffer, is

given optimal conditions for activity and consequently covers the effect of the alkaline or the bone phosphatase. Some types of saliva do not show hydrolyzing power at pH 9.4 but this does not mean, necessarily, that the bone phosphatase is totally absent from the saliva. It means, rather, that the local or individual conditions of hydrolysis are immediately neutralized. Possibly individual factors of solubility may be active, so that the enzyme is present in the desmo-form or in the lyo-form (Soluble or bone-bound enzymes).

The Mg. activating influence on the salivary phosphatases. Some experiments performed by the author confirmed the results of Giri (13) which showed that the Mg. ion does not influence the salivary phosphatase at pH 5.5 but that a weak activation may be observed at pH 9.4.

TABLE XXII

Storage and determination of the activity of different acid and alkaline types of saliva
1 ccm. saliva + 4 ccm. substrate. Inc. 37°. 3 hours

	ACID TYPE OF SALIVA (pH measured 6.1–6.7)			ALKALINE TYPE OF SALIVA (pH measured 7.0–7.3)			SALIVA WITH TOLUENE
Material	pH 5.5	pH 9	Sharer Unit	pH 5.5	pH 9.4	Sharer Unit	pH 5.5
Fresh saliva	6.8	3.2	15	4.8	2.3	12.5	5.8
Stored saliva (2 days)	5.5	2.4	12.5	3.8	1.6	10	4.8
Stored saliva (6 days)	5.1	1.6	7.5	3.2	1.1	7.5	3.6

TABLE XXIII

Phosphorus level of saliva at different pH

pH	P mg%	pH	P mg%
4.4	2.8	7.2	3.2
5.5	5.2	8.4	2.7
6.1	4.8	9.4	2.3

TABLE XXIV

Relationship of heating to phosphate level

	pH 9.4 mg% P	pH 5.5 mg% P
Fresh saliva	2.2	4.3
Saliva heated to 50′ (30 min.)	1.8	2.1
Saliva heated to 55′ (30 min.)	1.5	0.0

Destruction by Heat. The above experiments indicate that the alkaline phosphatase is more resistant to heating than the acid phosphatase. (Table XXIV)

The influence of substrate concentration on the activity. This problem has already been examined by previous investigators, including Giri (13) and Stein (24). The optimal activity of the salivary acid phosphatase appears at

a somewhat higher concentration than is in the case with the alkaline phosphatase for which the alkaline concentration is estimated at 0.025 mol. solution (0.5% to 1%) of sodium-glycerophosphate.

The cleaning by dialyse. According to Giri (13) dialyzed saliva should be more durable than that which is not dialyzed. This fact cannot be confirmed by experiments of the author.

The hydrolysis of the other substrates. (*Presence of pyrophosphatase.*) Demuth (6) and Giri (13) have demonstrated a well pronounced hydrolysis of hexosdiphosphate indicating the behavior of salivary phosphatases toward other substrates. The problem of hydrolysis of pyrophosphate seems to be more uncertain among the investigators. A distinct pyrophosphatase activity can be observed in the saliva under the experimental conditions used (Table XXV).

Activation by addition of different effectors. Some experiments were performed with different substances added to the saliva. The lactic acid, nitrate and cadmium seem to promote phosphatase activity, while fluoride seemed to have an inhibitory effect, especially on the acid phosphatase.

TABLE XXV

Hydrolysis of substrates. (*4 ccm. m/200 sodiumpyrophosphate with citrate buffer pH 5.5 + 1 ccm. saliva.*)

MEAN OF 25 CASES	INC. 3 HOURS P MG%	INC. 15 HOURS P MG%
Fresh saliva	2.6	10.4
Stored saliva (3 days)	3.0	12.2
Stored saliva (4 days)	2.4	9.8

The enzymic hydrolysis of the complex phosphate of saliva

As demonstrated by the previous experiments, the saliva contains some low molecular acid soluble phosphate compounds. An attempt was made to use these as a substrate for an enzymatic hydrolysis. Robinson has indicated a technique for procuring a solution of the acid soluble phosphates of the saliva. This technique, with some modifications, was used for saliva: To 5 ccm. of freshly collected whole saliva 5 ccm. of 1% trichloracetic acid was added. The mixture was vigorously shaken and filtered. To the filtrate a little phenolphtaline was added and thereupon drop by drop, 10% sodium hydroxide until a faint pink color appeared. The presence of the trichloracetic acid ion does not check the activity of the enzyme. Four ccm. of the solution was used for analyzing the enzymatic activity, 1 ccm. of buffer solution being added just before the experiment. As enzyme material, 1 ccm. extract from gingival tissues or from the periodontal membrane, was used. By using concentrated extract from pig's kidney the activity is promoted. To complete the above experiment, it is necessary to use freshly collected saliva, otherwise the self-hydrolysis of the substrate will cover the effect of the added enzyme. (Table XXVI)

The synthesizing effect of the salivary phosphatases

By analyses of some salivary material at different hours it is often observed that the inorganic phosphorus present shifts in value when the saliva is left at room temperature and under the influence of the air. A lowering of the inorganic value can only be explained by the synthesizing qualities of the saliva.

A. *In vitro experiments on the synthesizing salivary phosphatase. Technique.* The saliva was collected in the morning and divided into 2 portions. From these portions, after respectively 2, 4 and 8 hours, 2 ccm. were taken out for analysis: 1 ccm. for estimation of the total phosphorus of saliva and 1 ccm. for estimation of inorganic phosphorus. The portions were left at room temperature and exposed to the influence of the air (Table XXVII).

TABLE XXVI

Enzymic hydrolysis of organic salivary phosphate. (4 ccm. substrate + buffer solution + 1 ccm. enzyme-extract. pH 9.4. Inc. 3 hrs.)

EXTREME VALUES OF 25 EXPERIMENTS		MG% P	ENZYME
Case no.	Years		
2	18	3.1	Periodontal-extract
8	12	5.0	Kidney-extract
16	7	6.8	Peridontal-extract
24	11	1.4	Gingival-tissue-extract
7	8	2.8	Gingival-tissue-extract

TABLE XXVII

Synthesis by storage of the saliva

AVERAGE OF 25 EXP.	TOTAL ACID SOL. MG%	INORG. P. MG%	ORG. ACID	
			sol. mg%	pH
After hours				
0	18.1	14.9	3.2	6.7
2		10.6	7.5	6.9
4		9.8	8.3	7.0
8		14.2	3.9	6.6

In certain types of saliva (especially alkaline), a distinct increase of the organic phosphate by means of the synthesizing effect of the saliva can be observed during the first 4 hours. Later the phosphorus content is lowered on account of hydrolysis, which covers the effect of the synthesis. Alkaline buffer solution and a little fluoride is able to promote the synthesis.

The synthesis may be due to the effervescence of the carbon dioxide of the saliva and the pH shift which follows in the alkaline direction. Furthermore, the admission of the oxygen from the air and the lowering of the temperature from body to room temperature must be taken into consideration. Similar experiments with blood have been performed by Lawaczek (16) and have

shown that conduction of air or oxygen through the blood promotes synthesis. It is probable that the synthesis is due to a bacterial fermentation, as it is checked by addition of toluene.

It can hardly be lactic acid fermentation in this case, as such fermentation is greater at body than at room temperature. Lactic acid changes the hydrogen-ion concentration to the acid side, and in this way promotes hydrolysis as it can be observed by the long standing of the saliva without toluene. Probably synthesis is due to putrefaction of saliva with formation of ammonia. This, in combination with the effervescence of the carbon dioxide would give an immediate alkaline reaction to the saliva. In any case, the synthesis depends

TABLE XXVIII

Effect Storage of the Saliva after Admission of Carbon-dioxide

STORAGE	MIN.	TOTAL ACID SOL.	INORG. P. MG%	ORG. P. MG%	pH
Fresh saliva	0	19.2	17.2	2.0	6.9
Saliva + carbon-dioxide	120		18.4	0.8	6.6
Saliva without carbon-dioxide	120		16.4	2.8	7.1

TABLE XXIX

Effect of addition of tributyrin solution

AFTER HOURS	TOTAL P.	INORG. P.	ORG. P.	pH
0	17.8	15.2	2.6	7.1
2		14.1	3.7	7.1
4		13.0	4.8	7.2

TABLE XXX

AVERAGE OF 20 EXPERIMENTS	P MG% AFTER		
	0 hours	6 hours	12 hours
Phosphate + glycerin + saliva	14.6	11.4	10.2
Phosphate + glycerin + boiled saliva	14.6	14.6	14.7

on the alkaline reaction of the saliva. The development of ammonia in the saliva can be demonstrated by the pH level and by the Arne Hansens ammonia analysis (11).

The role of the carbon-dioxide in the hydrolysis can be demonstrated by a control experiment (Table XXVIII). In the carbon-dioxide treated saliva, no synthesis can be observed.

An augmentation of the alcoholic component of the saliva may be due to an increased intake of fatty substances and by the splitting of the fats into glycerin and fatty acids by salivary lipase.

As demonstrated by Scheer (22) and Eggers Lura (8) the lipase is quite active in the saliva.

Technique. Equal parts of a saturated tributyrin solution is added to the saliva and left at room temperature. Samples for analysis are taken out immediately and after 2 and 4 hours the values are corrected for the tributyrin addition (Table XXIX). The disengagement of the glycerin seems to promote the synthesis in vitro.

In accordance with the law of mass effect, every synthesis or hydrolysis of the esters in vitro must result in an equilibrium between esters and water, acid and alcohol. Provided that the necessary excess of the alcoholic component is present, a catalytic ester synthesis should be possible in vitro with the synthesizing salivary phosphatase as the catalysator (Table XXX).

In the majority of samples a distinct lowering of the content of inorganic phosphorus could be observed even after 6 hours of incubation and at room temperature. In some cases a better effect was obtained by addition of periodontal membrane extract to the tested mixture.

As the fluoride seems to have a retarding effect on the hydrolizing salivary phosphatase, an experiment was done with the addition of sodium fluoride

TABLE XXXI

Effect of fluoride

4 ccm. glycerin + 2 ccm. saliva + 0.5 ccm. sec. sodium phosphate ($\frac{1}{15}$ mol) + 2 ccm. buffer-solution pH 9.4 + 0.5 ccm. sodium fluoride (0.5%)

AVERAGE OF 10 EXPERIMENTS	MG% INORG. P AFTER		
	0 hours	6 hours	12 hours
Saliva + the test mixture	14.6	5.7	5.2
Boiled saliva + test mixture	14.6	13.9	14.1

(Table XXXI). The addition of the fluoride promotes the synthesizing activity of the saliva, while the hydrolyzing effect is checked.

DISCUSSION AND CONCLUSIONS

Since, in the present work, the phosphorus content of the saliva as well as the values for salivary phosphatases are expressed in mg. %, all shifts in the concentrations are directly comparable with the standard errors of the mean. Any difference is considered significant when it exceeds 2 times the value of the standard error of the mean.

The greatest differences between the salivary phosphorus values can be attributed to the varying phosphorus content of the diet with a rather high degree of certainty. In comparison to this diurnal variations, conceivably due to the varying bacterial content of the saliva, are relatively slight.

The greatest standard deviations of the mean for the organic fractions (3.1 mg. % for an average of 4.9 % for organic acid soluble phosphorus) cannot, for the present, be related to any known causes.

Through enzymatic activity, 0.5 to 2.3 mg. % inorganic phosphate of the saliva may be liberated in 1 hour, and as this is more than 4 times the error of

mean, the phosphatase activity must be considered significant. However, it is necessary to take into consideration that the capacity of the phosphatases for hydrolysis, which is in itself a variable which depends on the pH and the concomitant substances of the saliva. When the experimental material has been stored and left for some length of time, the synthesizing activity of the salivary phosphatases has to be taken into consideration.

Through an experimental series it has been possible to demonstrate differences in the inorganic phosphorus values amounting to 4 to 6 mg. % when the saliva is left at room temperature from 2 to 4 hours. This corresponds to about 2 times the standard deviation of the mean on the average value for inorganic phosphorus (3.4 mg. %). Although these differences are balanced to some extent on leaving the saliva longer, for the present they must be considered as the most natural explanation for the strikingly great differences in the normal values for the salivary phosphorus encountered heretofore in dental literature. In further analyses of the salivary phosphorus this source of error should be eliminated by carrying out the analyses immediately after collecting the saliva.

SUMMARY

1) In analyses of all phosphorus fractions, except in total phosphorus, rather significant shifts in the phosphorus concentration resulting from the activity of the phosphatases, and resulting indirectly from the influence of the concomitant substances on these phosphatases, should be considered.

2) When the saliva is left for some time, these shifts become particularly pronounced, as the enzymes are more resistant to storage than the phosphorus fractions. Hence the phosphorus analysis should be carried out immediately after the collection of saliva.

REFERENCES

1. Adamson, K. T., *Austral. J. Exper. Biol. Med. Science* **6**: 215, 1929.
2. Axmacher, T.: *Bioch. Zeit.* **248**: 231, 1929.
3. Becks, H., *Proc. Soc. Exp. Biol. Med.* **26**: 93, 1928–29.
4. Becks and Wainwright: *J. D. Res.* **14**: 387, 1934.
5. Becks and Wainwright: *J. D. Res.* **17**: 197, 1938.
6. Demuth, F., *Bioch. Zeit.* **159**: 415, 1925.
7. Clark and Shell, *Dental Cosmos.* **69**: 500, 1927.
8. Eggers-Lura, H., Undersogelser over Spyttets Enzymer. Kbn. 1944, Edition Munksgaard (Copenhagen).
9. Eggers-Lura, H., *Schweiz. Monats. f. Zahnheilk*, **56**: 39, 1946.
10. Eggers-Lura, H., *Schweiz. Monats. f. Zahnheilk*, **56**: 691, 1946.
11. Eggers-Lura, H., *Schweiz. Monats. f. Zahnheilk*, **57**: 33, 1947.
12. Entin and Geikin: *Deutsch. Monats. f. Zahnh.* **46**: 867, 1928.
13. Giri, K. S., *Bioch. Zeits* **285**: 386, 1936.
14. Glock, Murray and Pincus, *Bioch. Journal* **28**: 720, 1934.
15. Kay, H. D., *Phys. Rev.* **12**: 384, 1932.
16. Lawaczeck, *Bioch. Zeit.* **145**: 360, 1924.
17. MacFarlane, Patterson, Robinson: *Bioch. Jour.* **28**: 720, 1934.
18. Matthis, H., *Zeit f. Stomatolog.* **32**: 1188–1248, 1934.

19. Price, W. A., *Dental Cosmos.* **74:** 1139, 1932.
20. Price, W. A., J. A. D. A. **23:** 417, 1936.
21. Sharer: *Journal Dairy Science* **31:** 21, 1938.
22. Scheer, K., *Klinisch. Wochens.* **7:** 163, 1928.
23. Smith, G., *Australian Jour. exp. biol. Med. Science* **7:** 44, 1930.
24. Stein, K. S., O. M. Kontrol. af pasteur. Maelk. Disputats Kbn., 1936.
25. Valloton Ch, *Schweiz. Monats. f. Zahnh.* **52:** 512, 1942.
26. Wainwright and Becks, *J. D. Res.* **18:** 1, 1939.
27. Weber, R., *Zeits. f. Stomatolog* **28:** 233, 1930.
28. Youngburg, G., *J. D. Res.* **12:** 267, 1932.
29. Zander, H. A., *J. D. Res.* **20:** 261, 1941.

FACTORS INFLUENCING ORAL GLUCOSE CLEARANCE[1]

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In a previous study (1) we observed marked variations in the clearance from the mouth of glucose ingested in different foods and confections. Because of these inconsistencies a preliminary experimental investigation of this aspect of oral physiology was attempted.

The need for information on the subject is apparent if we agree that dental caries is related to retention of refined carbohydrates about the teeth and their subsequent breakdown by oral bacteria. Accordingly, those forms of carbohydrate which are cleared most slowly would have the most harmful effects on the teeth and should be avoided.

Included among the more probable factors influencing the clearance of glucose from the mouth are its physical nature and the manner in which it is consumed. Therefore it was decided to compare the clearance from the mouth of glucose taken in liquid and different solid forms. The solid glucose could be eaten in a granular mass, dissolved by sucking a wafer of pure glucose, and chewed in a resistant mass such as is provided by confections and chewing gum. It was felt that such an approach would give information indicating the fate of sugars used according to our present dietary habits. The selection of glucose for this study is not meant to imply that this carbohydrate is of greatest importance in caries etiology. This sugar was chosen primarily because it can be detected by reliable quantitative methods and was most suitable for our experiments.

EXPERIMENTAL

Six young adults (3 males aged 22–33, 3 females aged 22–27) were used as test subjects. To determine the base salivary glucose level (control value), initial samples of unstimulated saliva were collected from each patient at least 1 hour after the last food was eaten. Experimental salivary samples were taken at 2, 9, 16, and 30 minutes after the beginning glucose ingestion. The glucose was administered to each participant in 500 mg. quantities in the following forms (1) a glucose wafer to be held in the buccal vestibule and dissolved by sucking (2) a glucose cube to be eaten at the normal rate (3) 4 cc. of an aqueous solution of glucose to be rinsed in the mouth for one minute prior to swallowing (4) a gum base to be chewed for the entire experimental period. The first 3 glucose preparations listed were prepared in our laboratory and contained as much glucose as is usually found in a section of commercial chewing gum.

[1] This study was supported by a grant from the American Chicle Company. Received for publication February 27, 1947.

RESULTS

Typical findings of glucose clearance tests are presented in Table I. The figures reported are the averages of tests made on a group of 6 subjects. Repetition of the procedures gave essentially the same results. In general there was good agreement in the rate of clearance of the various supplements in 5 of the 6 participants. In a sixth subject who wore a removable partial denture, the disappearance of glucose was uniformly slower. At each time interval the highest values were encountered when glucose was sucked in wafer form. When glucose was eaten in cake form the 2 minute salivary level was in excess of 1000 mg%. However, the return to normal was more rapid than where glucose was given in concentrated aqueous solution. The lowest initial value (2 min.) was found in the case of the gum base glucose. Although the gum was chewed for the entire 30 minutes the saliva showed no evidence of added glucose after approximately 20 minutes of chewing.

TABLE I

Mg % of glucose in saliva at intervals after using 500 mg. of glucose

HOW USED	TIME IN MINUTES				
	0	2	9	16	36
Eaten (Cake)	18	1425	68	20	14
Sucked (Wafer)	18	3304	1860	1125	229*
Rinsed (Solution)	21	832	105	25	17
Chewed (Gum Base)	23	725	204	144	27

*3 of the 6 subjects had not completely dissolved their wafer. The 30 minute average figure for those dissolving the wafer was 91 mg %.

COMMENT

The data presented possibly casts light on one important factor in the causation of caries, namely the period of time during which the oral concentration of soluble carbohydrates will be elevated following varying methods of use. Our findings deal only with the use of sugar in a pure form and do not necessarily forecast what will happen when sugars are combined with other foodstuffs having varying physical properties. However, results may have some practical implications, since they relate directly to some of the more common methods of sugar utilization; namely, in crunched and sucked candies, chewing gum and sweet beverages. A conclusion of particular interest is that chewing gum in spite of the incorporation of glucose in a solid and persistent base gives low glucose values after the first few minutes of use. This may be related to the findings of Knighton (2) and Turesky and Bibby (3) which showed that gum chewing was effective in removing micro-organisms from the mouth, and to our demonstration that it removes carbohydrate foodstuffs.

Unpublished data from our laboratory gives further support to this concept. We have measured the titratable acidity after 24 hours incubation at 38°C of 5 cc. samples of saliva before (control) and at 5, 15, 30, and 45 minutes after

beginning to chew gum.[1] The average titratable acidities for 8 experimental subjects was 0.4, 2.7, 1.8, 0.2, and 0.1 cc. of 0.025N acid for the control, 5, 15, 30 and 45 minute samples respectively. The reduced acid production in the 30 and 45 minute salivary samples indicates that the chewing had reduced individually or collectively the amount of available fermentable carbohydrate or the number of acidogenic organisms capable of affecting its breakdown. Another possible explanation would be that the vigorous mastication altered the nature of the salivary protein either by dilution or to the extent that they contained relatively fewer acidic groups capable of buffering activity.

SUMMARY

The clearance from the mouth of 500 mg. portions of glucose sucked, eaten, chewed and rinsed-swallowed has been compared in 6 experimental subjects. The clearance was found to be most rapid for the rinsed-swallowed and slowest for the sucked glucose. When glucose was administered in the form of chewing gum low salivary glucose values were recorded after the first several minutes notwithstanding the persistence of the base. Attention is called to the possible significance of the findings in relation to the causation of dental caries.

REFERENCES

(1) Volker, J. F. and Pinkerton, D. M., *J. D. Res.*, **24**: 203, 1945
(2) Knighton, H. T., *J. A. D. A.*, **29**: 2012, 1942
(3) Turesky, S. and Bibby, B. G., *J. D. Res.*, **23**: 51, 1943

[1] Dentyne, 2 sections.

ACID PRODUCTION IN SALIVA-CARBOHYDRATE MIXTURES[1]

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It is generally believed that the prolonged retention of refined carbohydrates about the teeth and their subsequent breakdown by components of saliva contributes to the causation of dental caries. In a previous study (1) we have shown that following the ingestion of certain foodstuffs the concentration of glucose in the saliva is greatly increased and that relatively high values may be observed for extended periods thereafter. These findings naturally gave rise to questions regarding the breakdown of glucose and other carbohydrates persisting in the mouth. Therefore, it seemed worthwhile to investigate. (1) the rate of breakdown of various mono, di and polysaccharides by saliva; (2) the influence of carbohydrate or saliva concentration on the velocity of saliva carbohydrate fermentation; (3) the comparative susceptibility of raw and refined carbohydrates to salivary action.

EXPERIMENTAL

Since the breakdown of carbohydrate by saliva is characterized by rapid acid formation, estimations of this phenomena provide a simple and direct measure of the effects of saliva on carbohydrates. In this series of experiments the progress of carbohydrate breakdown was followed by means of titrations with 0.025N sodium hydroxide, using phenolphthalein as an indicator, and by pH determinations on a Coleman potentiometer. The test mixtures were incubated at 38°C and fresh unstimulated human saliva or the sedimentary portion thereof was used in all instances. Because of the implied relationship between acid formation in the mouth and dental caries, and the belief that this takes place more rapidly in mouths with active dental caries, saliva from caries susceptible individuals was selected for our studies.

1. *The Rate of Breakdown of Various Mono, Di, and Polysaccharides by Saliva.* 5 cc. aliquots of pooled saliva from patients with active caries were added to 2½ cc. of 10% solutions of various carbohydrates. (Because of the limited solubility of starch, 5 cc. of 5% starch was substituted.) The pH of the mixtures was measured at 30 minutes, 1, 2, 3, and 4 hours at which time the titratable acidity was determined. With each series of tests a similar glucose saliva mixture was run and saliva without added carbohydrate was also incubated to serve as an additional control.

Repeated incubation of saliva without carbohydrate failed to produce significant quantities of acid. In saliva-glucose mixtures the pH fell steadily from

[1] This study was supported by the American Chicle Company. Received for publication February 27, 1947.

the initial value of approximately 6.8 to a terminal pH of 4.5 at the end of 4 hours. The final titratable acidity generally approximated the equivalent of 6 cc. of 0.025N NaOH. The results given with fructose, sucrose, white dextrin and soluble starch were essentially the same as those observed with glucose. (Table I) Two pentoses, arabinose and xylose, both showed complete lack of acid production. The phosphorylation of fructose (commercially prepared fructose mono-phosphate and fructose di-phosphate) did not accelerate its reactivity. Yellow dextrin gave indication of being approximately 25% less susceptible to acid production than white dextrin.

2. *The Influence of Carbohydrate or Saliva Concentration on the Velocity of Saliva Carbohydrate Fermentation.* The influence of carbohydrate concentration on the acid-producing capacity of saliva was investigated by incubating mixtures containing varying concentrations of glucose and pooled saliva from carious persons. The mixtures were made by adding equal quantities of saliva and varying dilutions of glucose and contained between 100 mg% to 10,000 mg% of

TABLE I

*Acid formation in saliva carbohydrate mixtures**

	pH OF MIXTURE AT VARIOUS TIME INTERVALS						TERMINAL TITRATABLE ACIDITY
	min.	*min.*	*hr.*	*hrs.*	*hrs.*	*hrs.*	
Substrate	0	30	1	2	3	4	c.c./.025 N acid
Glucose	6.8	5.7	5.0	4.5	4.4	4.4	5.8
Sucrose	6.8	5.7	5.0	4.5	4.4	4.4	5.8
Starch	6.8	5.2	4.7	4.5	4.4	4.4	7.1
Fructose	6.9	5.5	4.9	4.6	4.4	4.3	6.0
White Dextrin	6.8	6.1	5.0	4.5	4.3	4.3	5.3

* 2.5 c.c. of 10% glucose, sucrose, fructose, and white dextrin and 5 c.c.of 5% starch plus 5 c.c. of pooled saliva.

the hexose. Determinations of pH were made each 30 minutes during a 3 hour period. At the end of this time the acid formed was titrated with sodium hydroxide.

No appreciable difference in acid production could be detected between 500 and 10,000 mg% solutions. Acid production in the 100 mg% mixtures proceeded at a reduced rate during the first 30 to 60 minutes and ceased thereafter. Analysis of these mixtures for reducing substances indicated that the cessation of acid production was the result of the glucose being completely utilized during the test period. (Table II) When sucrose was substituted in the saliva mixtures, results similar to those noted with glucose were observed.

The influence of dilution of salivary factors on carbohydrate breakdown was investigated by comparing acid production of the supernatant and sedimentary fractions of centrifuged pooled saliva. If 5 cc. of 10% glucose or sucrose were added to the same volumes of sediment and supernatant saliva, pH determinations made at 5, 15, 30, 45, and 60 minutes showed that acid production could

be detected in the former within 5 minutes but was not appreciable in the latter after 60 minutes incubation. At the end of an hour the pH of the supernatant mixture was ordinarily 7.0 and that of the sediment mixture 4.0. It seems apparent, therefore, that the ability of saliva to break down carbohydrate is dependent principally on the relative concentration of sediment contained in the saliva.

3. *The Comparative Susceptibility of Raw and Refined Carbohydrate to Bacterial Action.* Using the techniques previously described, the rate of acid formation was compared in pooled saliva to which various crude and refined carbohydrates were added. Cane sugar in 5 different stages of refinement from raw cane juice (freshly pressed from cane in our laboratory) to the ordinary crystalline table sugar showned no difference in acid production. Other tests with honey, maple sugar and farina (directly from the Amazon valley) indicated that other raw carbohydrates produced acid as rapidly as refined glucose or sucrose when acted upon by saliva.

TABLE II

*The effect of carbohydrate concentration on acid production in glucose saliva mixtures**

INITIAL CONC. MG. % OF GLUCOSE	pH OF MIXTURES AT VARIOUS TIME INTERVALS							TERMINAL TITRATABLE ACIDITY
	min. 0	min. 30	min. 60	min. 90	min. 120	min. 150	min. 180	c.c./.025 N acid
0	6.8	6.2	6.2	6.2	6.2	6.2	6.2	2.0
100	6.8	5.8	5.3	5.1	5.1	5.1	5.0	3.1
500	6.8	5.7	5.1	4.7	4.6	4.4	4.4	5.9
1,000	6.8	5.8	5.0	4.6	4.5	4.5	4.4	5.7
10,000	6.8	5.9	5.1	4.7	4.5	4.5	4.3	5.9

* 5 c.c. of pooled saliva and 5 c.c. aqueous solution of glucose.

COMMENT

These studies and those previously reported by Muntz and Miller (2) indicate that starch and sucrose are broken down by saliva at approximately the same rate and to the same extent as is glucose. This suggests the possibility that these poly and di-saccharides may not necessarily be converted to glucose during their degradation. Doudoroff's (3) evidence that sucrose may be phosphorylated directly by bacteria supports this idea. Other support is found in our inability to detect the production of reducing substances in saliva sucrose mixtures at a rate sufficiently rapid to account for the observed acid production. It is possible that an analogy can be drawn between the phosphorylation of glycogen (animal starch) and the breakdown of plant starch in the presence of salivary enzyme systems.

The rapid acid production that occurs when there is an increase in the relative amount of salivary debris (bacteria, cellular elements, mucin, etc.) in a saliva carbohydrate mixture supports the work of Stephen (4), who has demonstrated

that rapid acid formation occurs on tooth surface following glucose rinses. It appears likely that if salivary debris were concentrated to the extent that it is in undisturbed areas in the mouth that the addition of carbohydrates would give results comparable to those observed in his "in vivo" plaque studies of glucose fermentation.

The inability of raw carbohydrates to resist the attack of oral bacteria may at first be interpreted to mean that these substances are as destructive to the teeth as are their refined counterparts. However, Osborn, Noriskin and Staz (5) have reported that tooth surfaces exposed to mixtures of saliva and unrefined carbohydrates are more resistant to decalcification than those immersed in saliva-containing refined carbohydrates. Osborn, Noriskin and Staz suggested this phenomenon is the result of the refining processes removing constituents that are protection against solution of the enamel. Unfortunately our experiments cannot cast any light on this possibility.

SUMMARY

The unstimulated saliva of carious people has been incubated at body temperature with various carbohydrate solutions. The acid production in sucrose and starch saliva mixtures has been shown to be comparable to that of glucose. Two pentoses were found to be resistant to salivary action. It has been suggested that the breakdown of sucrose and starch in the mouth may not proceed through glucose but may be brought about by direct phosphorylation. Appreciable acid production has been observed to take place within 15 minutes in mixtures of glucose, sucrose and the concentrated salivary debris of carious persons. Various raw carbohydrates have been shown to be converted to acid by saliva at rates similar to those observed for refined sugars.

REFERENCES

1. Volker, J. F. and Pinkerton, D. M., *J. D. Res.*, **24**: 203, 1945.
2. Muntz, J. A. and Miller, B. F., *J. D. Res.*, **19**: 473, 1940.
3. Dourdoroff, M., *J. Biol. Chem.*, **151**: 351, 1943.
4. Stephan, R. M., *J. D. Res.*, **23**: 257, 1944.
5. Osborn, T. W. B., Noriskin, J. N., and Staz, J., *J. D. Res.* **16**: 165, 1937.

PREVENTION OF DENTAL CARIES BY BRUSHING THE TEETH WITH POWDERS CONTAINING FLUORAPATITE[1]

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Since the relation of experimental dental caries to fluorine was reviewed by Hodge and Sognnaes (1) references will be given mainly to our own work.

Fluorine interested the senior author in 1917 (2) when he observed that sea water (containing 1 part per million of fluorine) on evaporation would not completely redissolve, due partly to the insolubility of fluorides. Considering fluorine an essential element and finding it is lost from salt by refining, he began in 1919 giving rats sea water instead of salt. Fluorine was known to be a constituent of dental enamel and because of the calcium and phosphorus content he considered enamel to be essentially apatite. At his suggestion his associates confirmed this by X-ray spectogram (3).

With the aid of collaborators, the senior author showed (4) in 1921 that crystals of fluorapatite in saliva lost no weight in 3 months unless the pH was as low as 5. In 1923 he fed powdered rock phosphate (71.4% fluorapatite) to rats with beneficial results on their bones and teeth (5). Since the saliva contains calcium and phosphate ions and the pH is near neutrality and the dental enamel is essentially apatite, why does the enamel never add on calcium and phosphate ions and thus grow, but on the contrary always (when any change takes place) dissolve? A piece of polished marble in sterile sea water does not dissolve, but when immersed in the sea it becomes covered with organisms and etched by acids due to their metabolism underneath their attachments. The senior author showed that Tortugas sea water is supersaturated with calcite and aragonite but that neither calcite nor aragonite crystals would grow in this sea water unless they were absolutely clean (6). This may be due to interference of surface film with free diffusion. Freshly formed calcite crystals quickly came to equilibrium with sea water, which led to the idea of brushing the teeth in order to bring the enamel into equilibrium with the saliva. He first showed that an abrasive tooth powder of sharp calcite crystals caused no detectible abrasion of the enamel after 2 minutes brushing per day for 5 years (7). Brushing the teeth is no new idea since the tooth brush is reported to have been invented in China in 1498 and is figured in the Chinese Encyclopedia Lei Shu Ts' Ai T'u Hui in 1609. But the idea of subjecting a clean tooth to a saturated solution of fluorapatite was first published by us in 1942 (8, 9).

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BRUSHING ALBINO RATS' TEETH WITH POWDERED ROCK PHOSPHATE (71.4% FLUORAPATITE)

The brushing of rats' teeth with powdered Tennessee brown rock phosphate was continued 4 years with the litter-mate controls on the same diet. This diet was the Hoppert, Webber, Canniff (10) diet modified to lower its fluorine content. The diet was 59% cracked yellow corn, 30% low fluorine skim milk powder (N. Y. State) (11), 10% corn oil and 1% reagent sodium chloride. The diet was analyzed for fluorine by the method of McClendon and Foster (12) and usually fell within 0.3 and 0.5 parts per million. The rats were weaned at 21 days of age, put on the diet and half of each litter placed in a separate cage and their teeth brushed daily. The results were as follows:

NO. OF RATS		NO. OF DAYS ON DIET	CARIOUS TEETH PER RAT	
Brushed	Controls		Brushed	Controls
32	32	59–100	0.16	3
58	58	103–156	1.25	4

The rats were killed at the periods indicated and their jaws were placed under a binocular microscope and the carious teeth counted. Some of the teeth were later cleared in methyl salicylate and benzyl benzoate to verify the caries count, but since only comparative figures were needed this was discontinued. The caries were all in the molars. Fractures exposing the dentin were disregarded since it was found that they never became carious within 200 days and the incisors always had exposed dentin and were never carious. Fluorosis of the incisors was always present as indicated by bleaching along the daily growth lines or continuous bleaching. The caries were always in the sulci of the molars and if any very small ones were missed, the same was true of the controls. Multiple caries in a single tooth or confluence of caries was not recorded as only carious teeth were counted.

The rats learned how to resist the brushing process and since the number of carious teeth increased with the length of the experiment we concluded that we could not develop skill in brushing. This may be the reason we could never reduce the incidence of caries to zero.

The rats swallowed the phosphate rock and it increased the fluorine content of the enamel of their molars from 0.006 to 0.033% and the dentin of their molars from 0.0069% in the controls to 0.08% in those brushed 100 days. The fluorine content of the enamel of the incisors increased from 0.0047 to 0.014% and of the dentin from 0.0024 to 0.147% and their hardness increased so that they wore away more slowly than they grew in length.

A litter of 7 rats was divided into 2 groups, F and O. After 21 days each rat of group F had its teeth brushed with and ate about 0.1 gram rock phosphate per day and group O served as control. Group F (1 male and 2 females) raised a litter of young whereas group O (2 males and 2 females) had none. A litter of 8 was similarly treated and Group F (1 male and 3 females) raised 3 litters whereas Group O (1 male and 3 females) had none.

It appears that fluorine is necessary in the diet since rats raised on a fluorine-free diet grown by hydroponics lost their molar teeth down to the gingivae by caries in 48 days and died of malnutrition (13).

Litter mates had to be used for controls because the incidence of caries is not only influenced by genes but also by the diet of the mother. Rats on the low fluorine diet so seldom reproduced that we were forced to use rats whose mothers received rock phosphate, sodium fluoride or Purina dog chow which contained 10 parts per million of fluorine or 20 to 30 times as much as the low fluorine diet.

BRUSHING HUMAN TEETH WITH POWDERS CONTAINING FLUORAPATITE

The teeth of 150 medical students were examined for dental caries in the dental clinic of Hahnemann Hospital and the results tabulated as to which teeth (1–32) were involved. Of these students, 40 were given powdered Tennessee brown rock phosphate (71.4% fluorapatite) and told to brush their teeth with it daily. The first specimens of powder were collected by H. D. Ruhm from the exhaust ducts of the mill at Mt. Pleasant, Tenn., and 99.9% would pass a 300 mesh sieve. When this fine powder was exhausted, 100 lbs. of powder, 85% of which would pass a 300 mesh sieve (Ruhm's Lime Phosphate) was violently agitated in a barrel of water and allowed to settle. Only the top layer which contained less of the coarser particles was used. This rock phosphate contained 3.77% iron and was unpleasant to some students. Therefore an iron-free 'synthetic apatite' was prepared consisting of hydroxyapatite with a surface layer of fluorapatite (total fluorine content 0.25%). Thirty students were given the synthetic toothpaste and told to brush their teeth with it daily. At the end of a year the teeth of the students were re-examined and the new caries (developed within the year) tabulated. The 40 receiving the rock phosphate had developed 0.5 new cavities per man per year. The 30 receiving the synthetic product had developed 0.57 new cavities per man per year and the 80 controls had developed 1.5 new cavities per man per year.

ON THE NON-TOXICITY OF SWALLOWED FLUORAPATITE

During our experiments Dr. H. O. Calvery, Div. of Pharmacology, U. S. Food and Drug Administration, wrote us 3 letters claiming that fluorine in excess of 2 milligrams per day is a cumulative poison and is fraught with danger. When we received his first letter we were each taking 2.5 grams of rock phosphate per day containing 77.5 milligrams of fluorine. We did not stop the experiment but after 3 weeks reduced the daily intake to 1 gram containing 31 milligrams of fluorine. For 3 days one of us (F) took 1.5 grams of rock phosphate containing 46.5 milligrams of fluorine per day. The third day his urine contained 9.2 milligrams of fluorine and his feces 36.6, making a total of 45.8. His food and drink contained 1 milligram of fluorine, therefore his intake was 47.5, leaving an excess of only 1.7 milligrams for storage in bones and teeth.

One of us (M) had 2 molars extracted before taking rock phosphate and 2 molars extracted after taking 1 gram of rock phosphate per day for 6 months. The dentin of the control molars extracted before the experiment contained

0.019 and 0.033% fluorine. The molars extracted after taking a total of 183 grams of rock phosphate (5.7 grams of fluorine) contained 0.042 and 0.047% fluorine in the dentin indicating some storage. There was a maximum of 11 milligrams per day of fluorine in his urine but the feces were not examined since it was already determined that most of the fluorine goes out in the feces and the exact amount is made impossible to determine by inability to mark off the feces arising from one day's intake. We analyzed feces of 7 persons not taking rock phosphate; the average was 0.08 mg. per person per day, indicating that practically all the fecal fluorine after taking rock phosphate is due to the latter and probably represents unabsorbed fluorine. In fact, Machle, Scott and Largent (14) found only 0.039 milligrams per day in normal stool as average for 6 months experiment.

For a year each of us swallowed 1 gram per day of rock phosphate containing 31 milligrams of fluorine and for 3 years more one of us (F) swallowed 2 grams per week. The other (M) for 3 years more swallowed 1 gram per day of synthetic fluorapatite containing 2.5 milligrams of fluorine. Dr. N. Volney Ludwick repeatedly X-rayed the bones of our hands (and in 1 instance the spine) in comparison with aluminum plates 1, 2, 3, 4 and 5 mm. in thickness. No increased density or exostoses of the bones were observed.

Brun, Buchwald and Roholm (15) in the most complete study of fluorine poisoning in humans state that human osteosclerosis is produced by a daily absorption of approximately 25 milligrams of fluorine continuing over many years. Wolff and Kerr (16) analyzed the bones and teeth of a man who had worked 18 years in an atmosphere of rock phosphate dust that was 3.88% fluorine, in a superphosphate plant. The bones were thicker and heavier than normal, had chalky white areas and exostoses. The body of a lumbar vertebra contained 0.7% fluorine and the teeth 0.1% fluorine. Since at least 25% of the fluorine in rock phosphate is volatilized on the addition of sulfuric acid to make superphosphate this worker may have breathed in some hydrofluoric acid. Furthermore he may have acquired some fluorine during the first 30 years of his life, of which no medical history is given.

Tea is highest in fluorine of any food that has come to our attention. It contains about 0.05 to 1 mgm. per gram and 90% of it goes into the infusion (17). We bought some tea bags on the open market and analyzed them for fluorine. They contained 2.5 grams of tea each and 0.067 mgm. of fluorine per gram. A liter of tea made from tea grown (without insecticide) in Augusta, Georgia, contained 3 mgm. of fluorine. A native born British subject stated that a Briton might drink 10 cups of tea per day. This might contain 1.5 mgm. of fluorine. Glock, Lowater and Murray (18) analyzed ribs of Englishmen and found that the fluorine content increased with age and finally reached 0.3%. Why does not the fluorine in tea lower the incidence of dental caries in England? One suggestion is that the English do not give their babies tea and the other is that 1.5 mgm. per day is not enough to lower the incidence of dental caries. The amounts of fluorine used to prevent dental caries in experimental animals are far in excess of the amounts recommended for humans.

In an attempt to determine how the fluorine gets into tea we planted 2 tea

plants near Philadelphia but they did not live long so we planted 2 of its nearest relative (Camellia japonica). Dried leaves of one Camellia grown in ordinary soil (Lansdale silt loam) contained 0.01–0.12 mgm. of fluorine per gram whereas dried leaves of one Camellia grown in this soil fertilized with the Tennessee brown rock phosphate contained 0.4–1.4 mg. per gram.

McClure (19) found no deleterious effects of fluorine in recruits from the region of the air field at Lubbock, Texas, where the drinking water contained 5.1 mgm. of fluorine per liter. McClure does not state how much water was drunk per day. Allan Hemingway, in charge of the Laboratory of Biophysics, School of Aviation Medicine, Randolph Field, Texas, wrote us May 17, 1945, that each man drank 5–10 liters of water per day. This would bring the intake of fluorine to a figure which if continued for many years, according to Roholm, would cause osteosclerosis.

Shrewsbury, Hatfield, Doyle and Andrews (20) fed rock phosphate to lambs and attributing all its effects to its fluorine content found that 6 mgm. of fluorine per kilogram body weight per day depressed growth and produced fluorosis of the teeth. Goiter was also prevented, which they attributed to fluorine, though we believe that since the rock phosphate contained 10 parts per million of iodine, it was the iodine that prevented goiter. The senior author obtained 2 twin ewes 7 years old that had always grazed in the same pasture. One of them was fed 2 grams of Tennessee brown rock phosphate containing 62 mgm. of fluorine per day in the form of a sandwich. At the end of a year both were slaughtered and their bones and teeth examined in ultraviolet light which causes a more intense fluorescence of bones after fluorine poisoning. The fluorescence of the bones and teeth of the phosphate fed ewe was indistinguishable from those of the control. The crowns of 8 molar and premolar teeth in the upper jaw of the control had completely worn away leaving only the roots. Since we found that phosphate rock decreased the wear on rats' teeth this isolated instance is suggestive.

CONCLUSIONS

Brushing rats' teeth daily with powdered Tennessee brown rock phosphate decreases the incidence of dental caries on a cracked yellow corn, skim milk powder diet.

Except for the mottling of developing teeth, the toxicity of fluorine is greatly exaggerated. Most of the fluorine in rock phosphate is unabsorbed when it is swallowed by humans. There were less new caries in 1 year in the teeth of medical students brushing their teeth with powdered rock phosphate or 'synthetic fluorapatite' than in the controls.

APPENDIX ON PREPARATION OF "SYNTHETIC FLUORAPATITE"

Fluorapatite was made by setting up a 6 gallon crock filled with distilled water and fitted with an electric stirrer. Equivalent quantities of calcium hydroxide solution (lime water), phosphoric acid and sodium fluoride solution were added drop by drop from 3 burettes so as to correspond to the formula $CaF_2(Ca_3P_2O_8)_3$. The precipitate had to be separated by means of the centrifuge.

An easier way to prepare the toothpaste directly in 4 ounce jars (100 or so at a time) was

as follows for each jar: A 4 ounce glass jar was loosely filled with 36 grams of hydroxyapatite (calcium phosphate, tertiary, precipitated, U.S.P.). This is not labeled "apatite" but when it is made by precipitation and not heated above 110° C., it retains the apatite crystal lattice and the OH ions of the formula are derived from the water. Ten cc. of a mixture of equal parts of alcohol and glycerine plus 20 milligrams of menthol or thymol were added a little at a time from a burette. From another burette 10 cc. of 2% sodium fluoride was added, the cap was screwed on and the jar shaken. The function of the alcohol is to wet the dry powder more easily, the glycerine to prevent evaporation and the menthol or thymol to prevent the growth of mold. The fluoride entirely disappears from solution and it is believed that the hydroxyapatite takes up the fluoride ions by a process known to geologists as secondary enrichment. A common example is the formation of dolomite from limestone in contact with water containing magnesium ions. One magnesium ion replaces one calcium ion in the limestone. In a similar manner one fluoride ion replaces one hydroxyl ion in the hydroxyapatite. Gruner, McConnell and Armstrong[21] have figured a unit cell of the apatite crystal lattice indicating the positions where fluorine can substitute for hydroxyl ions. In their figure all these positions are occupied by fluorine ions.

Several persons prefer Ruhm's lime phosphate made by Ruhm Phosphate and Chemical Co., Mt. Pleasant, Tenn. It has been heated to 1000°F and altho sold as fertilizer, it is sterile.

REFERENCES

1. Moulton, F. R., *Dental Caries and Fluorine* (Washington, D. C., Am. Assn. Adv. Sc., 1946) p. 53.
2. McClendon, J. F., Gault, C. C. and Mulholland, S., The Hydrogen Ion Concentration, CO_2 Tension and CO_2 Content of Sea-Water, Pub. no. 251, Carnegie Ins. Washington, p. 39, 1917.
3. Taylor, N. W. and Sheard, C., Microscopic and X-Ray Investigations on the Calcification of Tissue, *J. Biol. Chem.*, **81**: 479, 1929.
4. Robb, E. F., Medes, G. and McClendon, J. F., A Study of Scurvy and its Bearing on the Preservation of the Teeth, *J. D. Res.*, **3**: 50, 1921.
5. McClendon, J. F., Rock Products in the Diet (Apatite for Teeth and Bones), *Rock Products*, **26**: 27, 1923.
6. McClendon, J. F., The Equilibrium of Tortugas Sea Water with Calcite and Aragonite, *Proc. Nat'n'l. Acad. Sc.*, **3**: 612, 1917.
7. McClendon, J. F., On the Equilibrium Between the Enamel of the Teeth and the Saliva, *Science*, **63**: 430, 1926.
8. McClendon, J. F., Foster, Wm. C., Ludwick, N. Volney, and Criswell, J. C., Delay of Dental Caries by Fluorine, *J. D. Res.*, **21**: 305, 1942.
9. McClendon, J. F. and Foster, Wm. C., Prevention of Dental Caries by Brushing the Teeth with Fluorapatite, *Federation Proc.*, **2**: 34, 1943.
10. Hoppert, C. A., Webber, P. A. and Canniff, T. L., The Production of Dental Caries in Rats Fed on Adequate Diet, *J. D. Res.*, **12**: 161, 1932.
11. McClendon, J. F., Foster, Wm. C. and Supplee, G. C., The Inverse Ratio between Fluoride in Food and Drink and Dental Caries, *Arch. Biochem.*, **1**: 51, 1942.
12. McClendon, J. F. and Foster, Wm. C., Determination of Dietary Fluorine, (Anal. Ed.) *Ind. Eng. Chem.*, **13**: 280, 1941.
13. McClendon, J. F., Fluorine is Necessary in the Diet of the Rat, *Federation Proc.*, **3**: 94, 1944.
14. Machle, W., Scott, E. W. and Largent, E. J., The Absoption and Excretion of Fluorides (I), *J. Ind. Hyg. Tox.*, **24**: 199, 1942.
15. Brun, G. C., Buchwald, H. and Roholm, K., The Excretion of Fluorine in the Urine of Cryolite Workers with Chronic Fluorine Poisoning, *Acta Medica Scan.*, **106**: 261, 1941.

16. Wolff, W. A. and Kerr, E. G., Composition of Human Bone in Chronic Fluoride Poisoning, *Am. J. Med. Sc.*, **195**: 393, 1938.
17. Reid, E., The Fluorine Content of Some Chinese Food Materials, Chinese *J. Physiol.* **10**: 259, 1936.
18. Glock, G. E., Lowater, F. and Murray, M. M., The Retention and Elimination of Fluorine in Bones, *Biochem. J.*, **35**: 1235–9, 1941.
19. McClure, F. J., Fluoride Domestic Waters and Systemic Effects I., *U. S. Public Health Reports*, **59**: 1543, 1944.
20. Shrewsbury, C. L., Hatfield, J. D., Doyle, L. P. and Andrews, F. N., Some Effects of Fluorine in the Nutrition of Sheep. *Purdue U. Ag. Exp. Sta. Bull.*, 499, 1944.
21. Gruner, J. W., McConnell, D., and Armstrong, W. D., The Relationship between Crystal Structure and Chemical Composition of Enamel and Dentine, *J. Biol. Chem.*, **121**: 771, 1937.

A NOTE ON THE MECHANISM OF FLUORIDE FIXATION[1]

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Experimental evidence has been offered by Ercoli (1) to support the hypothesis that fluoride replaces phosphate when a calcium phosphate, such as bone, is exposed to a fluoride solution. Several additional facts are known about the reaction between fluoride in solution and powdered bone. *First*, fluoride is rapidly removed from solution and firmly bound by a solid calcium phosphate (2). *Second*, the removal of fluoride from water by bone, dentin, enamel, or synthetic hydroxylapatite is a surface limited reaction described by the Freundlich adsorption isotherm (2, 3). *Third*, samples of powdered hard tissues exposed to fluoride ions exhibit thereafter a sharply reduced solubility in certain acid buffers (4). This effect has been attributed to a fixation of the calcium as CaF_2 and its retention in the powder particles even though phosphate has been liberated into the solution (5). The present experiment did not test this aspect of the reaction but was designed to determine whether, at or near neutrality where dissolution processes are not important, fluoride fixation by powdered bone simultaneously released phosphate into the solution.

Powdered bone marked with P_{32} was prepared by exposing 50 mg. samples of 60 mesh powder to solutions of approximately 10^{-4} M Na_2HPO_4 containing 1000 cpm of P_{32} per 1.6 mg. of P_{31}. This procedure permitted P_{32} from the solution (and P_{31} from the solution) to exchange with some of the P_{31} of the solid and thus to produce a marked or tagged bone phosphate. There are good grounds for believing that only the surface P_{31} atoms of the bone crystals took part in this reaction and that the 'crystals' are not the 60 mesh bone particles but rather the submicroscopic crystals of hydroxylapatite whose average length if of the order of 10^{-6} cm.

The P_{32} adsorbed on bone particles in this manner was readily removed or desorbed by reversing the procedure; i.e., by placing tagged bone powder in the same concentration of Na_2HPO_4 solution but one which contained initially no P_{32}. As is shown in *fig. 1*, the P_{32} rapidly exchanged so that in an hour most of it had left the bone and was in the solution. This did not represent an end state, the P_{32} was now in a new quasi-equilibrium, distributed between solid and solution. Replacement of the solution now containg P_{32} by a fresh sample of Na_2HPO_4 solution (unmarked) promptly resulted in a further exchange of P_{32} and a further decrease of the P_{32} content of the bone until another quasi-equilibrium was reached. This process was repeatedly carried out as shown in *fig. 1*.

[1] Received for publication March 18, 1947.

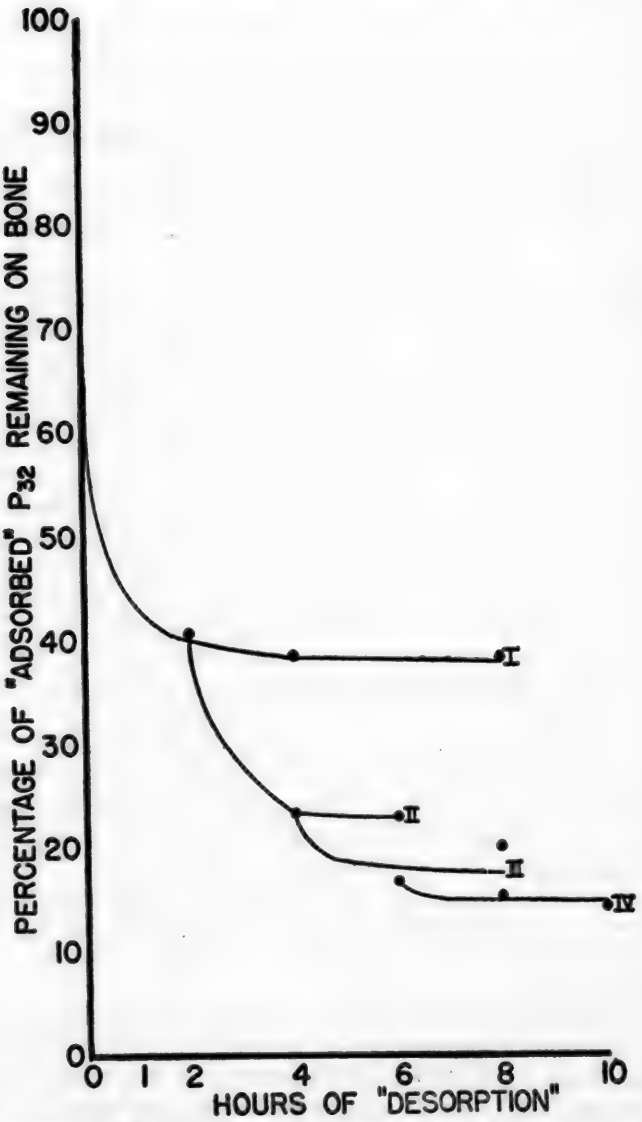

Fig. 1. Desorption of P_{32} From Bone. Bone marked with P_{32} and therefore originally containing 100% of the P_{32} when placed in a solution of 10^{-3} M Na_2HPO_4 solution for a period up to 8 hours lost more than half of the P_{32} adsorbed in a 1-hour period (Curve I). If at the end of 2 hours a fresh solution was used, additional loss of P_{32} occurred (Curve II). When this process was repeated (Curves III and IV), additional amounts of P_{32} were removed.

In preparing marked bone samples it was necessary to choose some standard time for the initial adsorption exposure because increasing the length of adsorp-

tion time decreased the amount of P_{32} desorbed in a given time (*fig. 2*). Since a large fraction of the P_{32} which would be adsorbed in 10 days had already exchanged even in a 1 hour adsorption exposure, the latter time was chosen for the fluoride tests.

A general description of the experimental methods has been published before (3). The principal modifications used in this study were those enabling adsorption and desorption exposures to be carried on for several days. In an open centrifuge tube at 40° C, considerable evaporation occurred in a few hours so that for longer experiments some sort of closed tube arrangement was required.

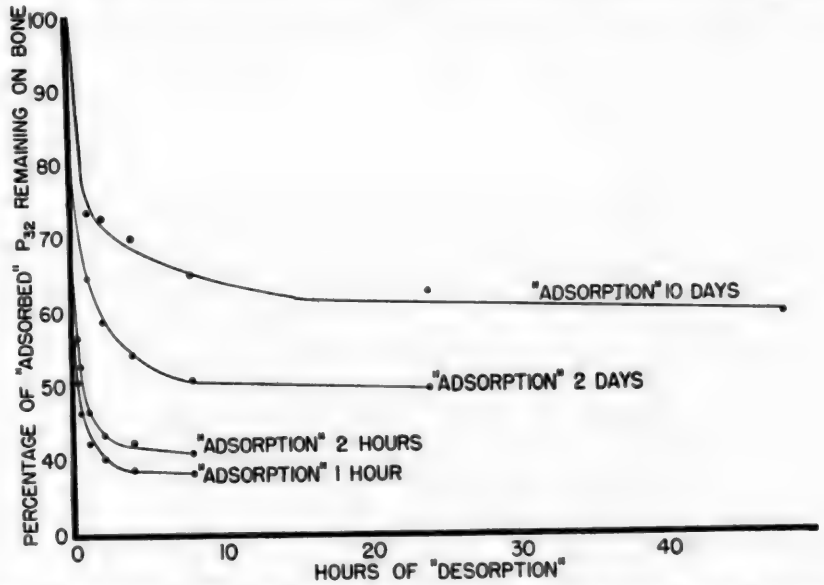

Fig. 2. Desorption of P_{32} From Bone After Various Adsorption Periods. When the bone was exposed for 1 hour to a solution containing P_{32} in Na_2HPO_4 and then a desorption procedure was carried out, the lowest curve was obtained. When the adsorption period was increased to 2 hours, less P_{32} was desorbed in a given time. Adsorption exposures for 2 days or for 10 days further decreased the rate at which desorption occurred.

The scheme finally employed kept the useful feature of using a centrifuge tube as a container. These tubes were placed, 10 per group, in specially built holders in which the hinged top maintained a firm pressure against the rubber stoppers thus pressing the tubes tightly into large cork stoppers serving as individual bases for the tubes (*fig. 3*). A cork ring around the upper portion of each tube held the tube from rattling. To maintain the selected temperature of 40° C., the shaking machine was placed in a thermostatically controlled room. The shaker provided an end to end rotation of the tubes several times per minute (*fig 4*). One long side of the shaker was hinged and could easily be opened to place tube racks in position. These procedures gave a satisfactory reproducibility.

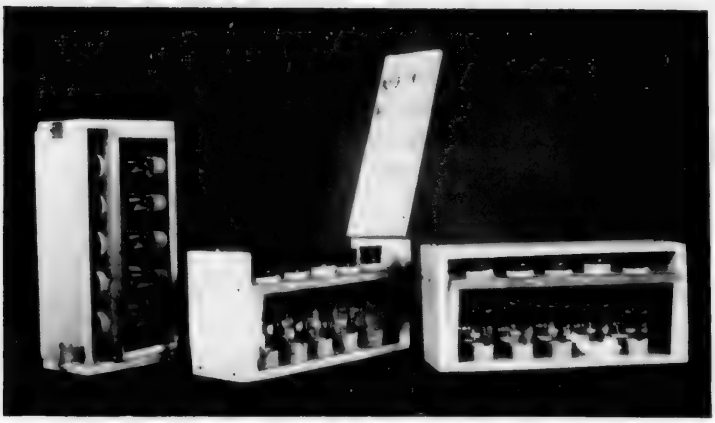

Fig. 3. Holders for centrifuge tubes. The 50 cc. centrifuge tubes were mounted in frames. Vibration was restricted by suitably prepared corks. Rubber stoppers were held in place by the hinged lids.

Fig. 4. Shaking Machine. The centrifuge tubes in their holders were placed in the shaking machine which slowly rotated the tubes end for end. The shaking machine was installed in a room thermostatically controlled at 40°C.

When tagged bone powder was exposed to a solution containing 1 part in a thousand of NaF, no more P_{32} was released to the solution than occurred when such a sample was exposed to distilled water (*fig. 5*). Treatment with 1:1000 NaF solution will reduce the acid solubility of powdered bone by nearly one-half (under specified conditions) so that fluoride undoubtedly was fixed by the bone. There is no evidence that this fixation was a simple exchange with phosphate; in fact, almost certainly phosphate exchange was *not* the mechanism of fluorine adsorption.

Since so little phosphate was removed by distilled water or by 1:1000 NaF solution, the suggestion was made that perhaps the fluoride might accelerate or increase P_{32} exchange from bone to solution when a large amount of phosphate

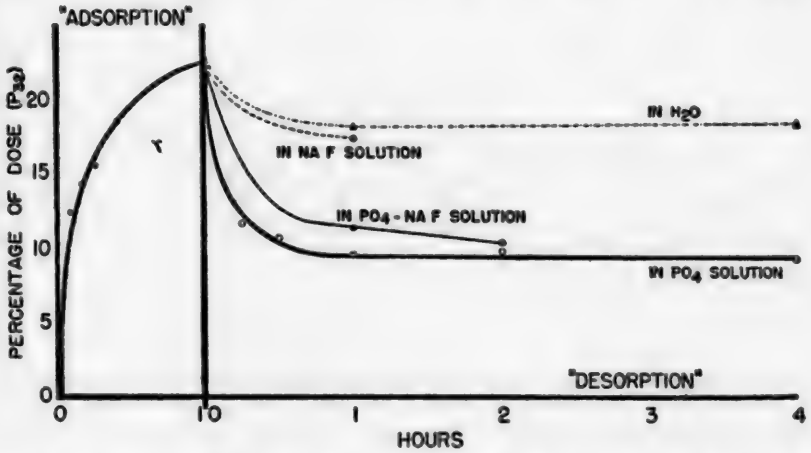

Fig. 5. P_{32} Desorption in Various Solutions. At the left the adsorption of P_{32} by powdered bone is indicated by the rising curve. After 1 hour adsorption, the P_{32} marked bone was exposed for time periods as indicated to distilled water and to a 1:1000 NaF solution, respectively. The rates of desorption of P_{32} were practically identical. In other experiments, the marked bone was placed in a solution of 10^{-3} M Na_2HPO_4 and a solution of the same concentration of phosphate containing in addition 1:1000 NaF, respectively. Again the rates of desorption of P_{32} were practically identical.

was exchanging. Accordingly marked bone samples were exposed to solutions containing 10^{-3} M Na_2HPO_4 *plus* 1:1000 NaF. Under these circumstances, no more P_{32} (and no less) went from bone to solution than when the powdered bone was treated with phosphate solution containing no fluoride.

Gerould has recently (6) suggested that the topical application of soluble fluorides, specifically sodium fluoride, produced insoluble calcium fluoride, a soluble sodium phosphate and probably some sodium hydroxide. Gerould stated that "the experiments with the pulverized enamel indicate that at least part of the soluble reaction products are leached out during the washing process". If such a reaction had occurred under the conditions of our experiment it seems plausible that phosphates should appear in the solution in greater

amounts than was observed. It is not doubted that Gerould produced a layer of calcium fluoride on powdered enamel exposed to 4% sodium fluoride solution; the mechanism of this reaction may or may not be the same as the mechanism of the reaction which we carried out. The failure to find additional phosphate in the solution in our experiments raises some doubt as to the interpretation of fluoride deposition offered by Gerould.

SUMMARY

1. When powdered bone samples marked with P_{32} were exposed to neutral aqueous solutions containing fluoride ions, fluoride was fixed by the bone. The mechanism of fluoride adsorption probably does not involve exchange with phosphate since no more P_{32} went from bone to solution than was found in the absence of fluoride ions.

REFERENCES

1. Ericoli, N., *Bull. Soc. Ital. Biol. Sper.* **13**: 755, 1938.
2. Smith, H. V., *Fluorine and Dental Health*, (Washington, D. C., Am. Assoc. Advanc. Science: 1942) p. 12 et seq.
3. Volker, J. F., Hodge, H. C., Wilson, H. J., and VanVoorhis, S. N., *J. Biol. Chem.*, **134**: 543, (1940).
4. Volker, J. F., *Proc. Soc. Exp. Biol. Med.*, **42**: 725, 1939.
5. Rae, J. J. and Clegg, C. T., *J. D. Res.*, **24**: 235, 1945.
6. Gerould, C. H., *J. D. Res.*, **24**: 223, 1945

A STUDY OF THE ACTION OF ANTIBIOTICS IN VITRO ON MICROORGANISMS ISOLATED FROM ROOT CANALS[1]

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Several reports have appeared on the treatment of infected root canals with penicillin. The earlier workers [Shaw, Sprawson and May (1), and Grossman (2, 3)] recognized that they had used too small a dose and explained unfavorable results on that basis. Buchbinder (4) used paper points which he calculated had soaked up 1,000 to 2,000 units of penicillin, or he injected oil containing 100,000 units/ml. directly into the canal with a syringe. In 33 of 36 cases treated, cultures from the canal became negative, though he was unable after 24 hours to demonstrate any penicillin in dressings which had originally contained 1,000 units. Stewart (5) forced penicillin into the canal with a syringe, using solutions containing 20,000 to 80,000 units in each milliliter and keeping as much of it as possible in the canal between sittings by packing with cotton and sealing with zinc oxide and eugenol. A hundred patients (112 teeth) were treated, and cultures from the canal were negative after 2 or 3 treatments in all but 2 cases. Stewart did not regard infiltrating above the apex to be necessary. In Bender's series (6) of 53 cases, the canal was negative on culture in 70% of the cases after one treatment. The treatment was accomplished by flooding the canal by means of a syringe and needle with 25,000 to 50,000 units several times during one sitting. He used both brain heart infusion and sodium thioglycollate broth as culture media and added clarase to inactivate any penicillin remaining at the time the culture was made.

Potkin (7) used paper points, adding just enough water to 100,000 units of penicillin to be completely absorbed by 200 paper points, hence each point represented a dose of 500 units. Unlike Buchbinder, Potkin found that the paper points still contained enough penicillin to inhibit *Staphylococcus aureus* after they had been in the canal 48 hours, even when the canal was putrescent. Potkin carried out a series of laboratory experiments in which he compared the inhibitory action of the paper points which were saturated with 500 units of penicillin and points which had absorbed 2 drops of various medicaments such as beechwood creosote and eugenol, using *Staphylococcus aureus* as test organism. From his photographs it appears that the penicillin point was as inhibitory

[1] The greater part of the data here reported is taken from the thesis submitted by the senior author in partial fulfillment of the requirments for the degree of Master of Science in Dentistry, Northwestern University, 1946. Presented to the Chicago Section, International Association for Dental Research, March 28, 1947. Received for publication April 16, 1947.

as the best of the medicaments and more so than most of them. Moreover, penicillin and beechwood creosote were apparently not incompatible; in fact, the mixture was more inhibitory to *Staphylococcus aureus* than either alone. Pear (8) tested both penicillin and streptomycin solutions, found that the two were not incompatible, and reported excellent results, on the basis of culture. In 34 cases treated with penicillin solution (5,000 units, injected "at or through the foramen"), all except 4 were negative after 1 to 4 treatments, and Gram-negative rods were present in these four. In 24 cases treated with streptomycin (aqueous solution containing 20,000 units/ml.), all were negative on culture after 3 treatments or less.

Some of the workers just quoted have attempted to estimate the effect of the antibiotic employed, using for this purpose impure cultures of oral bacteria. Grossman collected mouth washings in broth and inoculated plates of serum agar, then measured the zones of inhibition of growth in areas about cylinders of penicillin solution used as in the Oxford method of assay. Buchbinder plated cultures from the canal on brain heart agar and tested them with discs of blotting paper wet with penicillin solutions. We chose to isolate the microorganisms in pure culture and test them individually by standard methods for their sensitivity to penicillin and streptomycin, using standard strains of *Staphylococcus aureus* and *Escherichia coli* as controls in each test. We found, as have others before us (9), that the streptococci were the most frequently encountered microorganisms in the root canal, as elsewhere in the oral cavity. The mode of entrance of these organisms into the vital pulp has given rise to much speculation and controversy (10–15).

Bacteria have not been found to be sensitive to antibiotics in vivo if they have shown themselves to be resistant in vitro, hence a study of susceptibility of bacteria in vitro is ordinarily regarded as essential to provide a sound basis for clinical use and to estimate the dosage required (16). Although penicillin and streptomycin are relatively non-toxic, as compared with other chemotherapeutic agents, their use in suboptimal doses may defeat the very purpose for which they are used, by permitting the microorganisms at which they are aimed to become accustomed to them, gradually, until the amounts required to inhibit them in vivo are beyond those which can be attained (17, 18). Moreover, hypersensitiveness to penicillin is not unknown (19, 20), and its use in infections against which it cannot be expected to be effective may serve only to sensitize the patient, so that in subsequent emergencies he will have to be denied treatment with penicillin because of his acquired hypersensitiveness to it.

METHODS

Among patients referred for root canal therapy to the Department of Oral Pathology, 35 were selected who represented a fair distribution of various conditions, from simple carious exposures to putrescent pulps. Two cultures were taken from patients referred to the Oral Surgery Department when root canal therapy was not feasible.

Culture technique. After the tooth in question was anesthetized by the in-

filtration or block method, the field of operation was isolated by a rubber dam, and the tooth and surrounding area were swabbed with 75% alcohol and then painted with 3% tincture of iodine. All instruments used for excavation and enlargement of the pulp chamber, including chisels, excavator spoons, burs, broaches, and cotton forceps, were kept in individual glass containers in which they had been sterilized by dry heat at 180°C. for 1½ hours and were not exposed to air until immediately before use. The cotton pellets and paper root canal points were autoclaved in tubes stoppered with nonabsorbent cotton, and the same aseptic precautions were observed in their use.

The extirpated content of the root canal was dropped into a tube containing 10 cc. of fluid thioglycollate medium (21) in order to accomodate the growth of anaerobic as well as aerobic bacteria. A paper root canal point was introduced into the canal just long enough to absorb some of the exudate and accumulation of blood following the extirpation, and this was implanted in a second tube of thioglycollate broth. In putrescent pulp conditions, when mixed cultures were expected, duplicate points and tubes were used. The cultures were incubated for 24 to 48 hours at 37°C.

The bacterial growths obtained were streaked out on blood agar plates, which were incubated both aerobically and anaerobically, anaerobic conditions being obtained by the use of the Bray plate (22). Once a pure culture was isolated, it was identified as to genus, and further identification was postponed until tests of susceptibility to the antibiotics had been made.

Test for sensitivity to penicillin. The calcium salt of penicillin was supplied by Schenley Laboratories, through Prof. C. J. Farmer, of the Northwestern University Medical School, in vials of 200,000 units. The serial dilution method was used, and brain heart infusion broth was used for the solutions of penicillin, the serial dilutions, and the cultures, except in the case of the diphtheroids, which grew well only on veal infusion broth. Difco brain heart infusion broth, which contains 0.2% dextrose, proved entirely suitable for the test.

The concentration of penicillin in the first tube of each series was 10 units in 5 cc. (When unexpectedly high resistance was encountered, repetition of the tests was carried out with a series beginning with 50 units in 5 cc.) Each successive tube of the series contained half as much penicillin as the preceding one, and the last (11th) tube contained 0.009 units. The tubes were inoculated with 0.2 cc. of a 24 hour culture, the turbidity of which corresponded roughly to that of Tube 2 or Tube 3 of the McFarland nephelometer (600 million to 1 billion bacteria in 1 cc.). A parallel test was set up each time with *Staphylococcus aureus*, the standard "H" strain, obtained through the courtesy of the Department of Bacteriology of the Northwestern University Medical School. Readings of growth inhibition were recorded after an incubation period of 24 hours.

Test for sensitivity to streptomycin. The streptomycin was supplied by the Schenley Laboratories through Prof. Farmer, who also gave us a sample of standard streptomycin obtained from the Food and Drug Administration. The agar streak method (23) was used, with minor modifications as to the number of plates and units and the type of culture medium. The cultures were grown

on heart infusion broth except the diphtheroids, which were grown on veal infusion broth, and the agar used for the test was heart infusion agar or veal infusion agar. Seven dilutions of streptomycin were prepared, (in heart infusion broth except for the testing of diphtheroids), as follows:

1000 units in 1 cc.
500 units in 1 cc.
250 units in 1 cc.
150 units in 1 cc.
100 units in 1 cc.
50 units in 1 cc.
10 units in 1 cc.

A series of 7 Petri plates received the dilutions, 1 cc. in each instance, and an eighth plate 1 cc. of broth without streptomycin. The infusion agar was added in a quantity of 9 cc., so that the final concentration of streptomycin in each 1 cc. of agar in the plate was 100, 50, 25, 15, 10, 5 and 1 units. Each plate was marked into sectors, usually 6, so that several bacteria could be tested on a single plate. One sector of each plate was reserved for inoculation with a standard culture of *E. coli communis* of typical reactions, furnished by the Department of Medical Bacteriology. Three successive streaks were made in a given sector without recharging the loop. The results were recorded after an incubation period of 24 hours at 37°C. The control plate, without streptomycin, was inoculated in the same way as the other seven plates.

RESULTS OF SENSITIVITY TESTS

The total number of microorganisms isolated was 43, of which 20 were streptococci, 1 beta, 15 alpha, and 4 gamma. One pneumococcus was obtained, one Gram-negative rod, and one yeast. Several cultures of staphylococci (all *S. albus*) and micrococci were isolated, 3 diphtheroids, and 7 lactobacilli. The cultures grown under anaerobic conditions proved later to be facultative anaerobes and were thereafter grown aerobically. Eight cultures of alpha streptococci in the collection of the Dental Bacteriology Department, all of which had been isolated from dental infections, were also studied (alpha 18 to alpha 25).

The tests for sensitivity to the antibiotics are recorded in Tables I and II. There was no correlation between sensitivity to penicillin and sensitivity to streptomycin. In comparing the figures, it should be noted that the unit of penicillin is equal in terms of dry weight to 0.6 microgram, while 1 unit of streptomycin is equal to 1 microgram.

Among the streptococci (Table I), the highest resistance to penicillin was shown by the 2 cultures of enterococci, *S. liquefaciens* (alpha 14) and *S. fecalis* (alpha 18). A high resistance of *S. fecalis* to penicillin was recorded by MacNeal and Blevin (24), who obtained their cultures from the blood of patients with subacute bacterial endocarditis. It is interesting that our culture of *S. fecalis* was 4 times as resistant as any of our other streptococci, both to penicillin and streptomycin. The *liquefaciens*, on the other hand, was moderately susceptible to streptomycin. Hite and Hesseltine (25) also report resistance of enterococci

to penicillin. Three cultures of *S. liquefaciens* subsequently isolated in this laboratory had the same high resistance as No. 14.

TABLE I

Susceptibility of oral streptococci to penicillin and streptomycin

ORGANISMS TESTED	INHIBITION BY PENICILLIN UNITS IN 1 CC. 1 UNIT = 0.6 μg.	INHIBITION BY STREPTOMYCIN UNITS IN 1 CC. 1 UNIT = 1 μg.
Staphylococcus aureus H. Standard	0.062–0.125	
E. coli communis Standard		10 (15)*
#1 Alpha streptococcus	0.125	25
#2 Alpha streptococcus	0.500	10
#3 Alpha streptococcus	0.500	25
#4 Alpha streptococcus	0.250	10
#5 Alpha streptococcus	0.125	25
#6 Alpha streptococcus (S. bovis)	0.125	25
#7 Alpha streptococcus (S. bovis)	2.000	10
#8 Alpha streptococcus	0.250	25
#9 Alpha streptococcus	0.250	25
#10 Alpha streptococcus	0.062	50
#11 Alpha streptococcus	0.125	25
#12 Alpha streptococcus	0.125	50
#13 Alpha streptococcus	0.125	15
#14 Alpha streptococcus (S. liquefaciens)	2.00 –2.50	15
#15 Alpha streptococcus	0.062	25
#16 Alpha streptococcus (S. salivarius)	2.000	50
#17 Alpha streptococcus (S. mitis)	2.00 –2.50	15
#18 Alpha streptococcus (S. fecalis)	10.0	100
#19 Alpha streptococcus (S. equinus)	0.125	50
#20 Alpha streptococcus	0.062	15
#21 Alpha streptococcus	0.062	15
#22 Alpha streptococcus	0.125	25
#23 Alpha streptococcus (S. mitis)	0.062	15
#24 Alpha streptococcus (S. mitis)	0.062	15
#25 Alpha streptococcus	0.062	5
#1 Gamma streptococcus	2.000	10
#2 Gamma streptococcus (S. bovis)	0.062	25
#3 Gamma streptococcus (S. bovis)	0.125	10
#4 Gamma streptococcus	0.125	15
#5 Gamma streptococcus	0.062	15
#1 Beta streptococcus	0.125	15

* One sample of streptomycin was used in most of the tests, and 10 units of this sample inhibited *E. coli*. For the tests of alpha streptococci 18–25 and lactobacilli 5–7 the sample used inhibited the standard *coli* in a dose of 15 units.

Three other cultures, one of which proved to be *S. salivarius*, one *S. mitis*, and one *S. bovis*, showed considerable resistance to penicillin, but only the *sali-*

varius was also resistant to streptomycin. Alpha 19 (*S. equinus*) was susceptible to penicillin but resistant to streptomycin. Except for these 6 cultures, the

TABLE II

Susceptibility of various oral microorganisms to penicillin and streptomycin

ORGANISMS TESTED	INHIBITION BY PENICILLIN UNITS IN 1 CC. 1 UNIT = 0.6 μg.	INHIBITION BY STREPTOMYCIN UNITS IN 1 CC. 1 UNIT = 1 μg.
Staphylococcus aureus H. Standard	0.062-0.125	
E. coli communis Standard		10 (15)*
#1 Staphylococcus albus	0.062	5
#2 Staphylococcus albus	0.062	10
#3 Staphylococcus albus	0.062	10
#4 Staphylococcus albus	0.062	10
#5 Staphylococcus citreus	0.062	5
Diplococcus pneumoniae	1.000	25
Gram positive coccus	10.0	100
#1 Micrococcus	0.031	10
#2 Micrococcus	0.062	50
#3 Micrococcus	0.125	5
#4 Micrococcus tetragenus	0.500	5
#1 Diphtheroid	0.500	10
#2 Diphtheroid	0.062	15
#3 Diphtheroid	0.250	10
Gram negative rod	Not inhibited	10
Yeast	Not inhibited	Not inhibited
#1 Lactobacillus	3.00	50
#2 Lactobacillus	2.50	10
#3 Lactobacillus	2.50	25
#4 Lactobacillus	5.00	25
#5 Lactobacillus	2.50	15
#6 Lactobacillus	2.50	25
#7 Lactobacillus	2.50	25
Lactobacillus acidophilus ATCC	5.00	10

* One sample of streptomycin was used in most of the tests, and 10 units of this sample inhibited *E. coli*. For the tests of alpha streptococci 18–25 and lactobacilli 5–7 the sample used inhibited the standard *coli* in a dose of 15 units.

remaining 22 (i.e., the great majority of this series of streptococci of oral origin) proved nearly, if not quite, as susceptible to both antibiotics as the standard cultures (staphylococcus "H" and *E. coli communis*).

The data regarding the other cultures isolated are recorded in Table II. The staphylococci and the micrococci behave like the standard strain of staphylococcus with regard to penicillin, and the staphylococci were also susceptible to streptomycin. One Gram positive coccus (unidentified) showed a rather high resistance to both antibiotics. The pneumococcus (identified as such by its solubility in bile; it was not virulent for mice) was rather resistant to penicillin. The Gram negative rod, which was not identified with certainty (it is perhaps an Alcaligenes), though resistant to penicillin, was as susceptible as *E. coli* to streptomycin. Only the yeast was not inhibited by either antibiotic in the highest amounts tested (10 units per cc. for penicillin and 100 units per cc. for streptomycin).

The sensitivity of lactobacilli to penicillin is not clearly defined in the literature (26, 27, 28). Our cultures proved to be resistant, as compared with the standard staphylococcus. No. 4, for example, was not inhibited by less than 5 units of penicillin in 1 ml., and the least resistant cultures required 2.5 units/ml. The lactobacilli, then, were 20 to 40 times as resistant to penicillin as the standard staphylococcus. Their resistance to streptomycin was moderate, with one exception, No. 1, which was not inhibited by less than 50 units/ml. A standard lactobacillus culture obtained from the American Type Culture Collection showed a high resistance to penicillin, 5 units/ml. being required for inhibition, but it was as susceptible to streptomycin as the standard *E. coli*.

In order to compare the action of penicillin on mixed cultures, 4 organisms, all obtained from the root canal of 1 patient, were introduced into a test tube in equal amounts, and 0.2 cc. of the mixture inoculated into a series of dilutions of penicillin. Inhibition was achieved by less than 2 units of penicillin in 1 cc. The cultures used were alpha streptococcus 16 (*S. salivarius*), a micrococcus, a pneumococcus, and a diphtheroid (Table III).

Another type of test with mixed organisms was done with Lactobacillus No. 1 and Streptococcus No. 15, 0.2 cc. of the mixture of the 2 cultures being inoculated into a series of dilutions of penicillin beginning with 20 units per cc. Even in the first tube, growth was not completely inhibited. A series carried out with Lactobacillus No. 2 and alpha streptococcus No. 17 (*S. mitis*) gave similar results. The pH of the control tube inoculated with lactobacillus and streptococcus was 6.7 after 72 hours, hence the fall in pH was not sufficient to account for the reduced activity of the penicillin (29). The tests were repeated, but the series was started with 1,000 units of penicillin in a volume of 5 cc., or 200 units per cc., and a control series was added with the standard lactobacillus and *S. mitis*. With the latter cultures inhibition of both organisms occurred with 50 units per cc., and subcultures yielded negative results, but 200 units were required for lactobacillus No. 2 in combination with streptococcus No. 15. It appears, therefore, that certain combinations of bacteria, such as may be present in the oral cavity, may be more resistant to penicillin than the pure cultures of the individual organisms.

IDENTIFICATION OF LACTOBACILLI AND STREPTOCOCCI

The lactobacilli, besides being checked on tomato agar plates for typical colony and microscopic morphology, were grown on 2% dextrose broth and the final pH checked after a period of incubation of 12 days. All gave a final pH of 3.6.

The beta streptococcus was tested with sera of Groups A, B, and C, with negative results. Other group sera were not available.

A good number of our cultures of non-hemolytic streptococci were not readily identified as to species. We used the criteria laid down in Sherman's monograph (30). In Table IV are recorded the reactions of 11 cultures which we were able to identify, together with the reactions of standard cultures as recorded by Sherman. The reactions of the remaining cultures are recorded in Table V.

TABLE III

Action of penicillin on mixed cultures

Equal amounts of: Alpha streptococcus #16 (S. salivarius) Micrococcus Diplococcus pneumoniae Diphtheroid	0.2 cc. of this mixture inoculated into a series of tubes of penicillin broth
Inhibition of these organisms occurred at 2 units per cc. of penicillin	
Equal amounts of: Lactobacillus #1 Alpha streptococcus #15	0.2 cc. of this mixture inoculated into a series of tubes of penicillin broth
There was no inhibition even at 20 units per cc. of penicillin	
Equal amounts of: Lactobacillus #2 Alpha streptococcus #17 (S. mitis)	0.2 cc. of this mixture inoculated into a series of tubes of penicillin broth
There was no inhibition even at 20 units per cc. of penicillin	

As has been stated, the original cultures of thioglycollate medium were plated out on 5% rabbit blood agar and single colonies transferred to Difco brain heart infusion made semisolid with 0.15% agar. The latter medium is favorable to the multiplication of both streptococci and lactobacilli, and it was found that a few lactobacilli, which might be easily overlooked, could eventually overgrow the streptococci, probably because of a fall in pH incompatible with continued life of the latter. This situation developed in the case of 3 cultures which appeared to be pure cultures of streptococci when first tested (alpha 2 and 3, and gamma 1). After storage in the refrigerator for 2 months, with 1 or 2 subcultures during that period, streaking on blood agar showed less marked greening in the case of the 2 alpha streptococci, and all 3 cultures were found to be practically pure cultures of lactobacilli, giving a final pH of 3.6 in 2% glucose broth. It

is interesting that the resistance of alpha 2 and alpha 3 (listed in Table II as Lactobacilli 5 and 6) to penicillin had increased 5-fold.

Subsequently the attempt was made to separate mixtures of streptococci and lactobacilli by plating on rabbit blood agar, without success. The lactobacilli grew well when the pH of the blood agar plate was as high as 8.0, and they also grew well on blood agar containing 0.02% sodium azide. The streptococci were readily eliminated in the natural course of events, as described in the fore-

TABLE IV

Reactions of non-hemolytic streptococci

	Growth on tomato agar pH 5.0	Growth in 6.5% NaCl	Growth at pH 9.6	Growth at 45°	Final pH in dextrose br.		Starch hydrolysis	Milk		Gelatin	5% sucrose blood agar	5% sucrose Y.E.T. br.	Lactose	Mannitol	Maltose	Inulin	Arabinose	Raffinose	Salicin	Sucrose
					6.2	7.4		A	C P											
Enterococcus **group**																				
S. fecalis‡	....	+	+	+		4.0	−	++	−	−	−	−	+	+	+	−u.	±	−u.	+	±
alpha 18	+	+	+	+	4.0	4.0	−	++	−	−	−	−	+	+	+	−	+	−	+	+
S. liquefaciens‡	....	+	+	+		4.0	−	+++	+	−	−	−	+	+	+	−	±	±	+	+
alpha 14	+	+	+	+	4.1	4.4	−	+++	+	−	−	−	+	+	+	−	−	−	+	+
Viridans **group**																				
S. equinus‡	....	−	−	+		4.0	±	−−−	−	−	−	−	−	−	+	−u.	−	−u.	u.+	u.+
alpha 19	+	−	−	+		4.2	−	−−−	−	−	−	−	−	−	+	−	−	+	−†	+
S. salivarius‡	....	−	−	±		4.0	−	±±−	−	+	−	+	−	+	+u.	−	+u.	−u.	+
alpha 16	+	−	−	+	4.0	4.0	−	++−	−	+	−	+	−	+	−	−	+	+	+
S. bovis‡	....	−	−	+		4.0	±	±±−	−	−	−	+	±	+	+u.	±	+	+	+
alpha 6		−	−	+		4.2	+	++−	−	−	−	+	−	+	−	−	+	+	+
alpha 7		−	−	±*		4.3	±	++−	−	−	−	+	+	+	+	−	+	+	+
gamma 2		−	−	±*	4.5	4.2	+	++−	−	−	−	+	+	+	+	−	+	+	+
gamma 3	+	−	−	+	3.9	4.1	±	++−	−	−	−	+	+	+	+	+	+	+	+
S. mitis‡	....	−	−	±		4.7	−	+±−	−	−	−	+	−	+	−u.	−	−u.	+	+
alpha 17		−	−	+		4.5	−	++−	−	−	−	+	−	+	−	−	−	+	+
alpha 23	+	−	−	−		4.3	−	++−	−	−	−	+	−	+	−	−	−	+	+
alpha 24		−	−	+		4.7	−	++−	−	−	−	+	−	+	−	−	−	+	+

* Grew when transferred after 72 hours to 37°.

† Would make it *S. ignavus* by Holman's classification.

u. = usually ‡ Reactions as recorded in Sherman's monograph.[39]

going paragraph, but the elimination of the lactobacilli from a mixture with streptococci was not successful.

Once it had been found that lactobacilli might be present, all cultures were streaked out on tomato agar. No other cultures of lactobacilli were detected by this procedure, but several cultures of streptococci were found to grow well on tomato agar at pH 5.0 and to produce colonies indistinguishable from the so called "ground glass" type of lactobacillus colony. We submitted tomato

agar plate cultures of lactobacilli and of our aciduric streptococci to 2 persons accustomed to estimating lactobacilli in saliva, and neither was able to differentiate with certainty, by colony morphology alone, between the aciduric streptococci and certain of the intermediate colonies of lactobacilli.[3] Hemmens, Blayney, Bradel and Harrison (31) have recently recorded having found aciduric streptococci in cultures from the oral cavity. Subculturing on 2% dextrose broth of pH 6.2 and thence to tomato agar should probably be done early in the course of identification of oral streptococci. Streaking of plates of 5% sucrose blood agar is also useful, since the discoloration of the blood by non-

TABLE V

Reactions of non-hemolytic streptococci

	Growth on tomato agar pH 5.0	Growth in 6.5% NaCl	Growth at pH 9.6	Growth at 45°	Final pH in dextrose br.		Starch hydrolysis	Milk		Gelatin	5% sucrose blood agar	5% sucrose Y.E.T. br.	Lactose	Mannitol	Maltose	Inulin	Arabinose	Raffinose	Salicin	Sucrose
					6.2	7.4		A	C P											
alpha 4....		−	−	−		4.5	−	+	+−	−	−	−	−	−	+	−	−	+	−	+
alpha 5....		−	−	−		4.4	−	+	+−	−	−	−	+	−	+	−	−	−	−	+
alpha 8....		−	−	+		4.4	−	+	+−	−	−	−	+ weak	−	+	−	−	−	−	+
alpha 9....		−	−	+		4.4	−	+	+−	−	−	−	+	−	+	−	−	+	−	+
alpha 10...		−	−	+	4.3	4.3	−	+	+−	−	−	−	+	−	+	−	−	+	+	+
alpha 11...		−	−	−	4.0	4.5	−	+	+−	−	−	−	+	−	+	−	−	+	−	+
alpha 12...		−	−	−	4.6	4.3	−	+	+−	−	−	−	+ slow	−	+ slow	−	−	+	−	+
alpha 13...	+	−	−	−		4.4	−	+	+−	−	−	−	+ slow	−	+	−	−	+	−	+
alpha 15...	+	−	−	−		4.4	−	+	+−	−	−	−	+	−	+	−	−	−	+	+
alpha 20...		−	−	+		4.5	−	+	+−	−	−	−	+	−	+	−	−	−	−	+
alpha 21...		−	−	−*		4.3	−	+	+−	−	−	−	+	−	+	−	−	+	−	+
alpha 22...		−	−	−		4.3	−	+	+−	−	−	−	+	−	+	−	−	+	−	+
alpha 25...		−	−	+		4.3	−	+	+−	−	−	−	+	−	+	−	−	−	−	+
gamma 4...		−	−	+		4.3	+	+	+−	−	−	−	+	−	+	−	−	−	−	+

* Grew when transferred to 37° after 72 hours.

hemolytic streptococci is enhanced on this medium; even the "gamma" type of streptococcus may show a green or brown discoloration. The growth of lactobacilli on blood agar, with or without the sucrose, shows a minimal discoloration; on plain blood agar the growth is indistinguishable from that of a gamma streptococcus.

The test on 5% sucrose blood agar was first carried out for the purpose of detecting *Streptococcus salivarius*. Alpha 16 was apparently a pure culture of

[3] There is of course no confusion between the typical smooth lactobacillus colonies and the colonies of streptococcus. It is the minute ("ghost") or intermediate ("ground glass") colonies of lactobacilli which are responsible for the confusion.

this species, the only one obtained in this series. Subsequent results with 5% sucrose blood agar as a medium for saliva cultures in our classes have shown that *S. salivarius* is a common oral form.

The 2 cultures of enterococci in our series (Nos. 14 and 18) were revealed by subculturing from the stock brain heart infusion to brain heart infusion containing 6.5% sodium chloride. The salt test for the recognition of enterococci was superior to that with broth of pH 9.6 in our hands, because the pH tended to fall rapidly. Also accurate setting of a hydrogen ion concentration of this alkalinity is not possible with the older pH meters. *S. liquefaciens* was readily distinguished from *S. fecalis* by its ability to liquify gelatin and partially peptonize milk.

S. equinus was suspected when alpha No. 19 failed to curdle milk, and other tests confirmed the identity of this culture.

All cultures of non-hemolytic streptococci were tested for hydrolysis of starch on Difco heart infusion agar plates containing 1% soluble starch. The most marked action on starch was obtained in the case of alpha 6, which in a week had produced marked clearing for a considerable area around the quarter of the plate on which it had been streaked. The other cultures which appear to have hydrolyzed starch, when the plates were flooded with 1% iodine in 2% potassium iodide, as shown by failure of the area beneath their growth to become blue, were alpha 7, gamma 2, gamma 3, and gamma 4. The first three appeared on further testing to be *S. bovis;* gamma 4 was not identified.

Since *S. mitis* is the most common species found in the oral cavity, according to the experience of others (32), we expected to find more of these than are recorded in Table IV. We also sought the "s.b.e." streptococcus (33) by making the test on tryptone-yeast-extract broth containing 5% sucrose, but no viscosity was observed.

Hemmens, Blayney, Bradel and Harrison (31) have also reported considerable heterogeneity among the nonhemolytic streptococci of the oral cavity in connection with their work with bacterial plaques.

DISCUSSION

The difficulty of evaluation of penicillin and streptomycin for use in dental infections at the present time is indicated by the results described. Many of the nonhemolytic streptococci were found to be susceptible to one or the other or both of the antibiotics, but some exhibited a considerable resistance, as compared with the standard staphylococcus. The resistance of mixtures of lactobacilli and streptococci emphasizes the importance of bacteriological control in root canal treatment with penicillin.

The outcome of penicillin treatment in mixed infections is rendered uncertain, not only by the variation in sensitiveness to antibiotics of different bacteria (and even of different strains of the same bacterium), but also by the widespread occurrence of organisms which produce an enzyme capable of destroying penicillin, in some cases within as short a period as 2 hours (34).

The sound procedure for evaluating the effectiveness of antibiotics is to de-

termine what organisms are present and their sensitivity to the antibiotic before clinical application. It is the conclusion of those testing the usefulness of penicillin in treatment of infected wounds (16) that complete bacteriological analysis is essential if conclusive results are to be obtained. Local application, as is used in root canal therapy, has the advantage over parenteral administration that higher concentrations of antibiotic can be applied to the site of infection; nevertheless it appears that less satisfactory results can be expected from local than from systemic application (29).

It is well to bear in mind that in the treatment of root canals bactericidal rather than bacteriostatic action of the therapeutic agent is desirable, since the root canal itself is devoid of blood circulation once its content has been extirpated.

CONCLUSIONS

Fifty-one cultures of oral microorganisms were studied for their resistance or susceptibility to the 2 antibiotics, penicillin and streptomycin. Twenty-eight of these cultures were streptococci, and most of the species of non-hemolytic streptococci recorded in the literature were represented in our series by at least one culture. The exception is the so called s.b.e. streptococcus of Niven, Kiziuta and White (33), which they found to be more frequently associated with subacute bacterial endocarditis than other nonhemolytic streptococci. Many of our nonhemolytic streptococci were not satisfactorily identified, but none showed the viscous growth on 5% sucrose tryptone yeast extract which is characteristic of the s.b.e. streptococcus. Of the 28 streptococci, only 6 showed marked resistance to penicillin or streptomycin or both. These were the 2 enterococci (*S. fecalis* and *S. liquefaciens*), *S. equinus*, *S. salivarius*, one culture of *S. mitis* and one of *S. bovis*. The *fecalis* was outstanding in its resistance to both antibiotics.

The lactobacilli, of which 7 cultures were isolated, showed considerable resistance to penicillin, and when lactobacilli and streptococci were mixed, the resistance of the mixture was very much greater than that of pure cultures of either organism.

REFERENCES

1. Shaw, F. E., Sprawson, E., and May H. B., Bacteriological examination of infected dental pulp canals and use of penicillin in pulp-canal therapy. *Brit. Med. J.*, 1: 551, 1945.
2. Grossman, L. I., Evaluation of antibiotic agents for root canal treatment. *N. Y. J. Den.*, 15: 233, 1945.
3. Grossman, L. I., Experimental and applied studies in electro-sterilization. *D. Cosmos*, 73: 147, 1931.
4. Buchbinder, M., Use of penicillin as a root canal treatment. *J. D. Res.*, 25: 13, 1946.
5. Stewart, G. G., Preliminary report on penicillin in root therapy. *J. A. D. A.*, 33: 1281, 1946.
6. Bender, I. B., Penicillin in root canal therapy. *J. A. D. A.*, 34: 99, 1947.
7. Potkin, N. N., Use of penicillin in treatment of root canal. *J. A. D. A.*, 34: 459, 1947.
8. Pear, J. R., Preliminary case reports and technic on the treatment of apical infections with penicillin and streptomycin. *J. Endodontia*, 1: 32, 1946.

9. Nichols, A. C., Virulence and classification of streptococci isolated from apical infections (preliminary report). *J. A. D. A.*, **13**: 1218, 1926.
10. Henrici, A. and Hartzell, T. B., The bacteriology of vital pulps. *J. D. Res.*, **1**: 419, 1919.
11. Fish, W. E. and MacLean, I., Distribution of oral streptococci in the tissues. *Brit. D. J.*, **61**: 336, 1936.
12. Tunnicliff, R. and Hammond, C., Presence of bacteria in pulps of intact teeth. *J. A. D. A.*, **24**: 1663, 1937.
13. Bulleid, A., Bacteriological studies of apical infection. *Brit. D. J.*, **52**: 65, 1931.
14. Hatton, E. H., Structural changes in periapical and other oral infectious processes and their significance. *J. A. D. A.*, **24**: 1749, 1937.
15. Appleton, J. L. T., *Bacterial Infection*, (Philadelphia, Lea and Febiger: 1944) p. 358.
16. Meleney, F. L., Penicillin in treatment of established surgical infections: Systematic study of 744 including 82 septicemias. *Ann. Surg.*, **124**: 962, 1946.
17. McKee, C. M. and Dunham, W. B., Development *in vivo* and *in vitro* of strains of organisms resistant to penicillin. *J. Bact.* **48**: 121, 1944.
18. Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C., Increase in resistance of tubercle bacilli to streptomycin: a preliminary report. *Proc. Mayo Clinic*, **21**: 126, 1946.
19. Eisenstadt, W. S., Hypersensitivity to penicillin simulating serum sickness. *Minn. Med.*, **29**: 689, 1946.
20. Dressler, S. and Dwork, R. E., Reactions to penicillin. Procaine hydrochloride intravenously in the treatment of reactions similar to serum sickness. *J. A. M. A.* **133**: 849, 1947.
21. The medium originated by Brewer (J. A. M. A., **115**: 598, 1940) as modified by the National Institute of Health for sterility tests. Obtained from the Baltimore Biological Laboratories, Baltimore, Md.
22. A modification of the dish devised by Spray (*J. Lab. and Clin. Med.*, **16**: 203, 1930). Subsequent experiments showed that this method is far less satisfactory than the use of the Brewer anaerobic jar.
23. Waksman, S. A. and Reilly, H. C., Agar-streak method for assaying antibiotic substances. *Industrial and Eng. Chem.*, **17**: 556, 1945.
24. MacNeal, W. J. and Blevin, A., Bacteriological studies in endocarditis. *J. Bact.*, **49**: 603, 1945.
25. Hite, K. E. and Hesseltine, H. C., Study of aerobic streptococci isolated from the uterus and vagina. *J. Inf. Dis.*, **80**: 105, 1947.
26. Hobby, G. L., Meyer, K., and Chaffee, E., Activity of penicillin in vitro. *Proc. Soc. Exp. Biol. and Med.*, **50**: 277, 1942.
27. Kolmer, J. A., Penicillin Therapy, (New York, Appleton Century Co.: 1945) p. 273.
28. Garrod, L. P., Laboratory control of penicillin treatment. *Brit. Med. J.*, **1**: 528, 1944.
29. Penicillin. Brochure by Merck and Co. (1945). See also Suppl. 1946.
30. Sherman, J. M., The Streptococci. *Bact. Rev.*, **1**: 3, 1937.
31. Hemmens, E. S., Blayney, J. R., Bradel, S. F., and Harrison, R. W., Microbic flora of the dental plaque in relation to the beginning of caries. *J. D. Res.*, **25**: 195, 1946.
32. Sherman, J. M., Personal communication.
33. Niven, D. F., Jr., Kiziuta, A., and White, J. C., Synthesis of a polysaccharide from sucrose by streptococcus s. b. e. *J. Bact.*, **51**: 711, 1946.

TOBACCO AND GINGIVITIS[1]

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I. Statistical Examination of the Significance of Tobacco in the Development of Ulceromembranous Gingivitis and in the Formation of Calculus

Bergeron, (1859) called attention to the significance of tobacco-smoking in ulceromembranous gingivitis. He did not state that smoking was of direct significance in the development of the disease, but that there might be a question of predisposing effect associated with use of large quantities tobacco. According to Bergeron, many authors had mentioned the significance of smoking in the development of ulcero-membranous gingivitis, but only a few have devoted themselves to a more thorough investigation of the problem. Smeltzer (1921) did not consider smoking of tobacco responsible for the development of ulceromembranous stomatitis. He allowed his patients to smoke during treatment and considered smoking no handicap to treatment. Hirchfeld (1929) assumed that there might be some connection between the greatly increased consumption of cigarettes in the United States and the frequency of ulceromembranous gingivitis. Stammers (1944) expressed the view that tobacco smoking is an etiological factor in the development of ulceromembranous gingivitis and that it produces its effect in two different ways: When an individual carries a cigarette or pipe in the mouth the lips are slightly separated and even a moderate smoker of tobacco will breathe through his mouth a considerable part of the day, and consequently will be more exposed to the development of gingivitis. Furthermore, he considered the smoke as having an irritating effect, per se.

The object of the present work is to study whether it is possible, statistically, to demonstrate any correlation between tobacco smoking and ulceromembranous gingivitis.

One thousand four hundred and thirty-three (1,433) Danish Royal Marines were subjected to dental examinations. At the same time the Marines were questioned about their daily consumption of tobacco in the form of cigarettes, cigars and pipe tobacco. For the purpose of judging the quantities of tobacco one pipeful of tobacco was considered equivalent to 1 cigarette, that is about 1 gr. of tobacco. In reality a new pipe may hold more, but by frequent use the capacity of the pipe is diminished and it would not be any gross error to put one pipeful as equal to 1 cigarette. All of the Marines were between the ages of 16

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and 28 and the consumption of cigars and cheroots was insignificant. The Marines were divided into the following 3 groups on the basis of their smoking of tobacco. 1) Non-smokers, 2) consumption of tobacco less than 10 gr. per day, 3) consumption of tobacco 10 gr. or more per day. The gingiva were classified into the following 3 groups. 1) Normal, 2) chronic simple marginal gingivitis, 3) ulceromembranous gingivitis.

The results appear in Table I. Seemingly, chronic marginal gingivitis is not affected by tobacco smoking, whereas, the incidence of ulceromembranous gingivitis is markedly different in non-smokers and smokers. While 1.5% of the non-smokers present ulceromembranous gingivitis, 10.7% of the Marines smoking more than 10 gr. of tobacco a day had this disease. Moreover, the number of Marines with normal gingivae declines from 33% in non-smokers to 22.3% in the heavy smokers.

TABLE I

Showing influence of consumption of tobacco on the incidence of gingivitis

	NON-SMOKERS	CONSUMPTION LESS THAN 10 GR. TOBACCO PER DAY.	CONSUMPTION OVER 10 GR. TOBACCO PER DAY.	TOTAL
Normal gingiva	65 33.0%	187 28.5%	129 22.3%	381 26.5%
Chronic simple marginal gingivitis	129 65.5%	432 65.8%	388 67.0%	949 66.2%
Ulceromembranous gingivitis	3 1.5%	38 5.8%	62 10.7%	103 7.2%
Total	197 100.0%	657 100.1%	579 100.0%	1433 99.9%

The question presents itself. Why do the cases of ulceromembranous gingivitis increase with an increasing consumption of tobacco, or what does the tobacco contain which makes the smoker more susceptible to ulceromembranous gingivitis? Several theories have been propounded, attempting to explain the harmful effects of tobacco on the mucous membranes of the mouth. Among the theories are: 1) Tar contained in the smoke has a direct irritating effect on the gingiva, thereby causing gingivitis, 2) The direct influence of the heat of the smoke is able to cause gingivitis, 3) Carbonmonoxide, pyridine and other poisonous substances contained in the smoke may have toxic effect on the gingiva, so as to predispose to gingivitis and 4) Nicotine gives rise to vascular spasms, and it is possible that the nicotine contained in the smoke causes a contraction of the capillaries in the gingiva, resulting in a poor nutrition of the gingiva and a diminished resistance to infection.

The data in Table II indicate that the deposition of calculus is proportional to the consumption of tobacco. With a greater amount of tobacco smoked, there

seems to be a greater chance of developing calculus. While 39.7% of the non-smokers are without calculus, only 17.5% of the persons smoking more than 10 gr. of tobacco a day are calculus free. Corresponding hereto there is an increase in the deposition of supragingival as well as subgingival calculus with arising consumption to tobacco. Even though it is possible to see cases of tobacco stains without deposits of calculus it is a natural thing to associate the increased

TABLE II

Showing influence of consumption of tobacco on deposit of calculus

	NON-SMOKERS	LESS THAN 10 GR. TOBACCO PER DAY.	MORE THAN 10 GR. TOBACCO PER DAY.	TOTAL
Minus calculus	58 39.7%	168 29.6%	84 17.5%	310 26.0%
Supragingival calculus	69 47.3%	315 55.6%	275 57.2%	659 55.2%
Subgingival calculus	19 13.0%	84 14.8%	122 25.4%	225 18.8%
Total	146 100.0%	567 100.0%	481 100.1%	1194 100.0%

TABLE III

Showing significance of the deposit of calculus in the frequency of gingivitis

	MINUS CALCULUS	SUPRAGINGIVAL CALCULUS	SUBGINGIVAL CALCULUS	TOTAL
Normal gingiva	291 50.0%	271 20.0%	21 4.2%	583 24.0%
Chronic simple marginal gingivitis	268 46.0%	949 70.1%	382 76.9%	1599 65.7%
Ulceromembranous gingivitis	23 4.0%	133 9.8%	94 18.9%	250 10.3%
Total	582 100.0%	1353 99.9%	497 100.0%	2432 100.0%

deposit of calculus in smokers with the retention possibilities which the tobacco stains present.

This statistical study indicates that consumption of tobacco is significant in the development of an ulceromembranous gingivitis and in the deposition of calculus. It would be of interest to be able to establish that the deposit of calculus was of significance in the development of ulceromembranous gingivitis. Table III shows the results on 2,432 Marines examined. In chronic marginal gingivitis as

well as in ulceromembranous there is a rising frequency according to the presence of supra-or subgingival calculus. At the same time it will be seen that among persons without calculus 50% have normal gingiva and among those who have supra or subgingival calculus there are only 20% and 4.2% respectively with normal gingivae.

It seems strange that subgingival calculus may occur in cases where the gingivae is normal. This can be explained as the result of calculus formation during a previous gingivitis, and after this gingivitis has healed the gingiva now presents a normal appearance with subgingival calculus.

On the basis of the results here reported nothing can be inferred as to the manner of action of the tobacco, but it is no doubt true that tobacco-smoking may play a role in the development of ulceromembranous gingivitis, and therefore smoking during treatment for an ulceromembranous gingivitis should be forbidden.

SUMMARY

On the basis of examinations of 1433 Royal Danish Marines, statistics show that the more one smokes the greater is the chance of developing an ulceromembranous gingivitis, and that the deposition of calculus is affected by the consumption of tobacco. From examination of 2432 Marines, deposition of calculus appears associated with development of gingivitis.

REFERENCES

Bergeron, E.-J, De la Stomatite ulcereuse des Soldats, Paris, *Labe:* p. 70, 1859.
Hirschfeld, K., *J. A. D. A.* **16:** 623, 1929.
Smeltzer, J. L., *J. A. D. A.* **8:** 924, 1921.
Stammers, F. A., *Brit. D. J.*, **76:** 147, 171, 205, 1944.

THE EFFECT OF AGE AND REGION OF BIRTH UPON THE RELATIVE NUMBER OF NAVAL PERSONNEL HAVING DENTAL PROSTHETIC REPLACEMENTS*

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INTRODUCTION

The Dental Division of the Bureau of Medicine and Surgery requested that a study be undertaken from which the needs of the Division and the dental status of naval personnel could be estimated (1). In accordance with their desire, the records of approximately 70,000 navy men volunteering in 1942 have been analyzed (2). In the first partial report of this analysis it was estimated that 12,100 dental officers were needed for 1 year to repair completely the teeth of 3,000,000 navy men (3). The second (4) and third (5) reports showed that persons born in the New England area have more cavities than those born in any other area and that they also have received the greatest amount of treatment. The west south central region, comprised of Arkansas, Louisiana, Oklahoma and Texas, was the lowest in amount of dental work required by each person and in the amount of work expended on him. The effects of age and region of birth upon cavities, missing teeth and fillings, also were considered in these reports. The object of the present study is to determine the effects of these same factors upon the relative number of persons provided with dental replacements.

SAMPLING PROCEDURE

The dental records used in the study were duplicates of those made at the time of the dental examination given at six recruit training camps, namely, Great Lakes, Norfolk, Parris Island, San Diego, Newport, R. I., and the Marine Corps Base, San Diego. The duplicate records were transferred to punch cards of which an example is given in an earlier report (3). In addition to the dental data, the card included the age of the man and the state in which he was born. The state of birth was later changed to region of birth in accordance with a regional division used in U. S. Public Health surveys.

The dental prosthetic appliances for which classification was provided are: full upper and lower, full upper only, full lower only, full upper and partial lower, full lower and partial upper, partial upper and partial lower, partial

* The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or the endorsement of the Navy Department. Received for publication March 18, 1947.

upper only, partial lower only, jacket crowns, post crowns, gold crowns, removable bridges, and fixed bridges.

The men whose records have been studied were volunteers and Fleet Reserves called to active duty. In 1942, the time of this survey, it was required that naval personnel have (a) 18 serviceable teeth including 2 opposing molars on either side; (b) good occlusion; (c) freedom from oral infection; and (d) suitable prosthetic replacements for any wide edentulous areas.

In the case of Fleet Reserves, the dental requirements were often waived, whereas the newly enlisted men all met the minimum dental standards. The punch card classification, unfortunately, did not separate those to whom waivers were given from those obliged to meet the dental standards. However, it is probable that the men in whose favor the dental requirements were waived do not represent more than 4% of the total sample, although they comprise 30% of some age groups. Wherever this lack of homogeneity undoubtedly obscures the effect of age and region of birth upon replacements, the data have

TABLE I

The actual and theoretical age distribution of the present sample of naval personnel

AGE	17–19	20–24	25–29	30–34	35–39	40–44	45 AND OVER
Actual	18,542	29,002	9,697	6,684	3,557	1,539	564
Theoretical	12,811	29,504	13,236	6,782	3,327	2,120	1,804

TABLE II

The actual and theoretical regional distribution of the present sample of naval personnel

	NE	MA	ENC	WNC	SA	ESC	WSC	M	P
Actual	6014	12972	7474	6502	11116	5747	10332	3672	5755
Theoretical	5465	13969	13544	6902	9065	4418	6233	2380	7601

been discarded. Such data are those on full upper and lower dentures, full upper, full lower, full upper and partial lower, full lower and partial upper dentures. These data merely show the relative number of each age group granted waivers.

The distribution of the sample with respect to age does not represent the age distribution of naval personnel in 1942 as well as one would like. The theoretical distribution of the sample and the actual distribution are given in Table I. The actual distribution shows tolerable agreement with the theoretical distribution in all but the 17–19 year age group and the 45-and-over age group.

The regional distribution of the sample is also weighted in favor of several regions and against others. In Table II the actual and the theoretical size of the samples from the different regions of the country are given. From an inspection of Table II it may be observed that the east north central region is not represented by a sufficient number of men, and the west south central has

too large a representation; the other discrepancies being less serious. The states included in each region are shown in *fig. 1*.

The failure of the actual distributions to conform with the theoretical distributions is due in part to the fact that the data from which the theoretical distribution was derived were not available at the time the sample was drawn. The distributions were computed from data published in 1944 by the Bureau of Personnel, Navy Department (6). The theoretical regional distribution is itself subject to error inasmuch as region of residence before enlistment rather than region of birth is the basis of the derived distribution. The failure of the actual distribution to conform to the theoretical distribution alters the mean values in a manner difficult to evaluate, but the forms of the curves very probably are unaltered.

Fig. 1. States in geographic regions

RESULTS

The effect of age upon replacements. In Table III are presented those replacements which are found in less than 4% of any age group. These replacements are partial upper only, partial upper and partial lower, one or more jacket crowns, one or more post crowns, and one or more removable bridges. The first 2 types of replacements show a continued increase with age; post crowns and jacket crowns seem to reach a maximum around 32–37 years of age, and are found less frequently at later ages. Partial lowers and removable bridges may show a similar maximum with the peak occurring possibly 5 years later than in the case of the post and jackt crowns.

In *fig. 2* those replacements found in more than 4% of some age groups are shown. One or more fixed bridges and gold crowns are plotted as a function of age. The fixed bridges reach a maximum around 37 years of age and then decrease as do the post crowns and jacket crowns. The gold crowns show a continuous increase with increase in age.

TABLE III

Per cent personnel of different age groups having indicated dental prosthetic replacement

REPLACEMENT	AGE IN YEARS						
	17-19	20-24	25-29	30-34	35-39	40-44	45 and over
Partial upper only	0.46	0.84	1.80	1.95	2.16	2.14	2.66
Partial upper and partial lower	0.07	0.19	1.01	1.38	2.05	2.40	3.55
One or more jacket crowns	0.45	1.1	1.4	1.7	1.7	1.5	1.3
One or more post crowns	0.12	0.32	0.62	0.91	1.4	0.91	0.71
One or more removable bridges	0.04	0.08	0.24	0.19	0.31	0.32	0.18

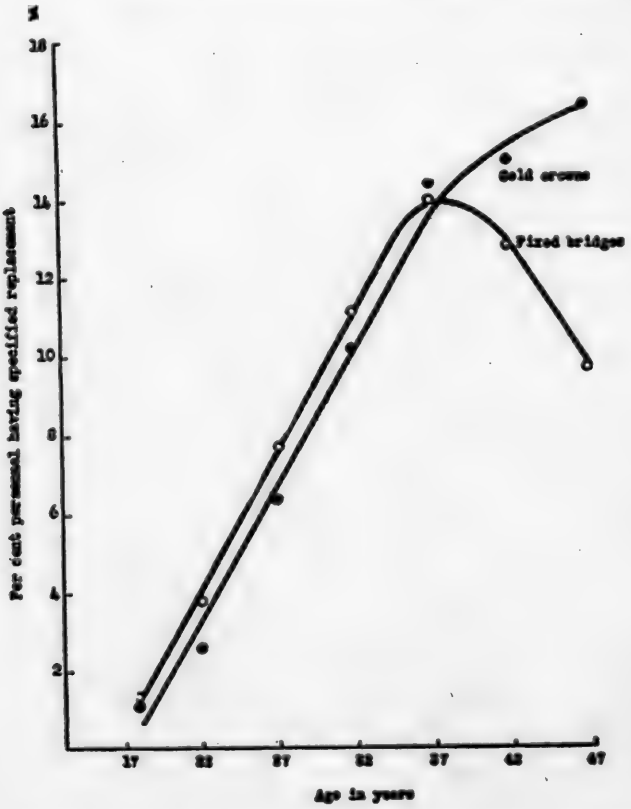

Fig. 2. Per cent personnel of indicated ages having fixed bridges and gold crowns.

The effect of region of birth upon replacements. It has been shown in the previous section that the relative number of men having dental replacements is a function of the age of the group. In comparing various regions of the country it is evident that the groups must have the same age distribution in

order to make the direct comparison between them valid. Unfortunately, the present sample age distributions of the various regions of the country are not the same, so it has been necessary to resort to an expedient whereby it is hoped that the groups were made equal.

Weights have been given to each age group by which the various ages were made equivalent to the age at which the maximum per cent was reached. The purpose of the weights is to minimize the age difference between regions. This was done by regarding the maximum per cent reached in the per cent personnel

TABLE IV

Adjusted rates per 1000 persons for the nine regions of the United States for fixed bridges and gold crowns

REGIONS	FIXED BRIDGES	GOLD CROWNS
New England	179.3	148.4
Middle Atlantic	163.6	196.0
East North Central	161.7	191.9
West North Central	153.1	166.3
South Atlantic	110.6	129.3
East South Central	114.0	132.1
West South Central	104.1	128.7
Mountain	109.9	143.8
Pacific	150.7	242.1

TABLE Va

Analysis of variance of rates per 1000 having 1 or more fixed bridges

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	VARIANCE
Region	8	46,029.27	5,753.66
Age	6	1,920.22	320.04
Error	48	62,958.07	1,311.63
Total	62	110,907.56	

$F_R = 5753.66/1311.63 = 3.39$ $P < .01$

age function (*fig. 2*) as one, and considering the other per cents as fractions of one. The per cents less than the maximum are made equal to it by multiplying the per cent by the reciprocal of the fraction proper to that group. The reciprocals obtained from the data of *fig. 2* constitute the weights for each age group. The efficiency of this method is indicated by the size of the age variance in Tables Va and Vb, where the age variance is smaller than the variance due to experimental error.

The 9 regions into which the United States was divided have been compared on the basis of fixed bridges and gold crowns. The weighted rates of these two replacements for each region are given in Table IV.

These weighted rates represent the number of persons per thousand having

the specified prosthetic appliances. The rate is for 37 years for fixed bridges and 47 years for gold crowns. That there are significant differences between various regions is evident from inspection of Tables Va and Vb, which present

TABLE Vb

Analysis of variance of rates per 1000 having 1 or more gold crowns

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	VARIANCE
Region	5	83,749.06	10,468.33
Age	6	878.24	146.37
Error	47	72,707.17	1,546.96
Total	61	157,334.47	

$F_R = 10468.33/1546.96 = 6.70$ $P < .01$

TABLE VI

Per cent of each age group having the indicated denture

DENTURE	AGE IN YEARS						
	17-19	20-24	25-29	30-34	35-39	40-44	45 and over
Full upper only	.59	.26	1.26	1.59	2.19	3.44	4.26
Full upper and partial lower	.02	.09	.60	1.05	2.42	2.47	3.90
Partial upper only	.46	.84	1.80	1.95	2.16	2.14	2.60
Full lower only	.02	.01	.01	.12	.25	.46	1.42
Full lower and partial upper	.00	.01	.05	.03	.17	.07	.53
Partial lower only	.08	.21	.57	1.17	1.38	1.69	.71

TABLE VII

Analysis of variance of upper and lower dentures

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	VARIANCE
Position	1	164.2610	164.2610
Type	2	36.6172	18.3086
Age	6	195.0592	32.5099
Interaction			
P × T	2	11.7826	5.8913
P × A	6	16.9520	2.8253
T × A	12	23.1446	1.9287
Error	12	17.9800	1.4983
Total	41	465.7966	

$F_p = 164.2610/2.1169 = 77.5051$ $P < .01$

the results of the analysis of variance (7) of the weighted rates for the 2 types of replacements.

The west south central region, namely Arkansas, Louisiana, Oklahoma and Texas, has the lowest number of persons with either fixed bridges or gold crowns.

This result is consistent with the general finding that the people of this section have better teeth than those of any other region of the country. The fact that these people have less dental attention than those of other regions is another factor bringing about the low rate found (5).

The replacement of upper and lower teeth. The present data lend themselves to a comparison of upper and lower dentures. The data used were obtained in part from those persons to whom waivers were given. There is no reason to believe that waivers were given more freely to those with upper replacements than to those with lower replacements, so it would seem that these data form an acceptable basis for a comparison.

Inspection of Table VI reveals that upper teeth are replaced more frequently than lower ones. The data presented in this table have been converted to the arc $\sin\sqrt{\%}$, and an analysis of the variance of these has been made. This analysis presented in Table VII shows that, independent of the particular type of denture, uppers are more common than lowers. Whether these replacements are more frequent because of aesthetic reasons or because upper teeth decay more rapidly than lowers will have to await further investigation.

SUMMARY AND CONCLUSIONS

The dental records of some 70,000 men entering the Navy in 1942, when rigid naval dental standards were still in effect, have been examined to determine the effect of age and region of birth upon the relative number of dental replacements. This sample unfortunately was not entirely confined to those persons meeting the naval dental requirements, but included members of the Fleet Reserve. Despite this lack of homogeneity in the sample, one may conclude that:

1. The relative number of personnel having either partial upper only, partial upper and partial lower replacements, or gold crowns, increases with age. Those having either jacket crowns, post crowns, removable bridges or fixed bridges, increase until approximately 35 years of age, and then decrease beyond that time. In this sample as high as 14% of the older age groups have fixed bridges and gold crowns. The other types of dentures mentioned are found in less than 4% of any age group.

2. Personnel in this sample born in the west south central region, i.e., Arkansas, Louisiana, Oklahoma and Texas, have fewer fixed bridges and gold crowns than those born in other regions of the United States. This may be due either to better teeth or to less dental care available and lower per capita income in this region than in others, as shown in previous reports (5, 8). It is obvious that results obtained on unscreened personnel cannot be determined from this sample.

3. Upper teeth are replaced more frequently than are lower teeth.

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REFERENCES

1. KNOX, A., Personal verbal communication to C. A. Schlack in Feb. 1942.
2. Project X-44, approved by Bureau of Medicine and Surgery 24 June 1942; subsequently changed to X-131 on 11 May 1943 upon transfer to Naval Medical Research Institute 24 Mar. 1943.
3. SCHLACK, C. A., RESTARSKI, J. S., AND DOCHTERMAN, E. F., Dental status of 71,015 naval personnel at first examination in 1942, J. A. D. A. **33**: 1141–1146, 1946.
4. SCHLACK, C. A., RESTARSKI, J. S., AND DOCHTERMAN, E. F., Dental status of 71,015 naval personnel at first examination in 1942. Age and regional distribution of persons with specific type dental defects. J. D. Res., **25**: 107–119, 1946.
5. SCHLACK, C. A., AND BIRREN, J. E., Time required to treat dental defects in 71,015 naval personnel, with regional and age variations, J. A. D. A., **33**: 1406–1418, 1946.
6. Yearbook of Naval Personnel Statistics, 1944, NavPers 15115 Bureau of Naval Personnel, Navy Department, Washington, D. C.
7. SNEDECOR, G. W., Statistical methods, 1946, The Collegiate Press Inc., Ames, Iowa.
8. Schlack, C. A., and Birren, J. E., Influences on dental defects in naval personnel, *Science*, **104**: 259–263, 20 Sept. 1946.

EXPERIMENTAL PERIODONTAL POCKETS IN DOGS[1]

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Methods and procedures for the treatment of periodontal disease have been prescribed for at least 3500 years. However, the method of treatment still remains controversial although many technics have been developed. To evaluate the merits of new procedures and the various technics, careful clinical study by experienced operators is necessary. This alone is not sufficient to obtain a complete picture of the result since histologic sections of the periodontium are also necessary. Obviously it would be difficult to obtain this material for sectioning from clinical patients, and therefore we are compelled to resort to laboratory animals with induced pathosis.

Pockets from 5 to 8 mm. in depth were produced in dogs with the method to be described. Some of these pockets have been under observation for as long as 5 months and show no signs of reattachment. This is probably sufficient time to determine whether the periodontal membrane will reattach itself of its own accord.

PROCEDURE

The dogs were anesthetized by intravenous injections of nembutal, 1 ml. for each 5 pounds of body weight. Because of the anatomical form of the crown and root, the canine teeth were found to be most suitable to retain the copper bands. They have little or no contact with the adjacent teeth and they are single rooted. These factors facilitated the placing and maintenance of the bands. Other teeth in the mouth were used, but best results with this technic were achieved with canine teeth. The condition of the gingiva and the depth of the gingival sulcus were examined and recorded. Photographs were taken and study models constructed at this time to supplement this record.

A Wagner inlay carver was forced down along the root surface for appromimately 8 mm., severing the periodontal membrane, and then with a lateral motion, the pocket was enlarged to a width of about 4 mm. If the pocket was left undisturbed, there was a connective tissue reattachment of the periodontal membrane with subsequent elimination of the pocket. To prevent reattachment, a copper band was used as a mechanical barrier and was placed between the alveolar bone and the root surface. The band was cut in such a manner that

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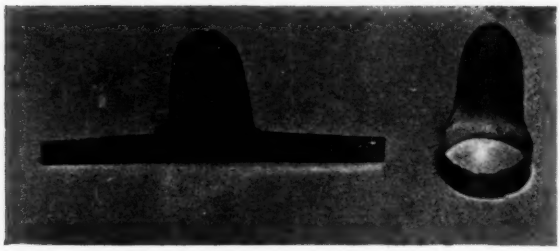

Fig. 1. Copper band on left shows method of cutting. Band on right has been adapted to root surface and cervical portion of tooth.

Fig. 2. Photograph showing bands in place on canine teeth of a dog.

Fig. 3. Decalcified section of a tooth that had band removed 150 days before the animals was sacrificed.

a tongue extended into the pocket (*fig. 1*). This tongue was pushed into the pocket formed by the Wagner carver, and the gingival surface of the band was burnished to the tooth surface. The band was then removed and the ends were soldered with low fusing silver solder.

The band was cemented to the tooth with zinc oxyphosphate cement, the excess being removed to decrease unnecessary irritation to the gingival tissues. The band itself caused some local irriation but this was kept at a minimum by having the band well adapted to the tooth surface. Generally the gingivae of the maxillary teeth showed a more acute reaction from the band than did the gingivae of the mandibular teeth. The reaction usually is accompanied by a 2 or 3 mm. recession of the gingiva in the region of the pocket. This is probably a permanent recession, as the gingiva thus affected on the experimental animals did not regenerate to its former contour.

After 15 days had elapsed from the time of cementation, the band was removed. Sections of tissue excised at this time from the pocket wall showed inflammatory cell infiltration and a few areas of young epithelium growing down the wall of the pocket.

The dogs were sacrificed 150 days after the bands were removed. Decalcified sections of the periodontium showed pockets extending an average 6 mm. along the root surface. There was a definite lining of epithelium growing down along the pocket but it was not attached to the root surface. Numerous round cells were observed below the epithelium.

SUMMARY

The technic described is a simple and positive means of producing typical periodontal pockets in experimental animals. Approximately 100 pockets were produced and observed for a period up to 150 days. Histologic sections at this time showed a pocket very similar to the pathologic periodontal pocket occurring in man.